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A STUDY OF THE TOXICITY AND SELENIUM CONTENT OF SELENIFEROUS DIETS: WITH STATISTICAL CONSIDERATION [1]

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Robinson's (1933) discovery of selenium in grains which Franke (1934) had found toxic greatly accelerated progress in the study of the disturbance colloquially known as the "alkali disease." Selenium is generally considered to be the toxicant in the foodstuffs but one can not overlook the possibility that molybdenum and tellurium (Beath, Eppson, and Gilbert, 1935), chromium, vanadium, and arsenic (Byers, 1934), and other poisonous metals may be taken up by plants in amounts which would render them dangerous when used as food for animals.

Franke and Potter (1935) fed selenium salts to albino rats and found the effects practically identical with those produced by seleniferous grains. Painter and Franke (1935) removed the selenium from toxic protein hydrolysates and Franke and Painter (1935) found the selenium-free product nontoxic when fed. Franke, Moxon, Poley and Tully (1936) produced monsters in chick embryos by injecting selenium salts into eggs. Some of these abnormalities were apparently identical with those Franke and Tully (1935, 1936) produced by feeding hens toxic grain. There was, however, a wider variation in types of monsters and greater involvement of organs when the natural toxicant was fed.

Due to these differences and apparent differences observed in gross pathology in rats fed experimental diets containing selenium, the question arises: Were the observed results due to the different selenium

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compounds or to additional effects of other poisons? Beath and coworkers (1934) and Draize and Beath (1935) observed different external pathology when different seleniferous plants were eaten. They attribute this to the different compounds of selenium and in some cases to the secondary action of other poisons.

Beath (personal communication) states that the toxicity of certain plants grown in Wyoming does not vary according to selenium content. This would be expected inasmuch as Beath, Draize, Eppson, Gilbert, and McCreary (1934) and Franke and Painter (1935, 1936) have shown that different forms of selenium occur in plants. Although it has not been conclusively proved that selenium is the sole toxicant, other toxicants, if present, may produce symptoms similar to those of selenium. Furthermore, they must have certain chemical properties in common with selenium.

When the first reports on the effects of toxic grains on animals appeared, the selenium content had not been determined quantitatively. The selenium content of the samples which have been collected and bio-assayed during the past six years of experimentation has now been determined. Selenium has been found in all samples which were found toxic.

If, by a comparison of the toxicity of the different samples, it is found that there is a difference in toxicity, other poisons than selenium or selenium compounds of different toxicity must be present. By a comparison of the toxicity of groups of diets it should be possible to ascertain whether or not specific kinds of cereals always metabolize selenium compounds of the same toxicity. If the toxicity of selenium salts added to an otherwise normal diet is included in this comparison, the relative toxicity of naturally occurring (organic) selenium and inorganic selenium may be determined.

There may be a seasonal variation in the toxicity of the samples. Steyn (1934) finds the presence of most plant toxicants profoundly affected by climatic conditions during the growing season. The metabolism of selenium compounds may also depend upon the growing conditions. If a seasonal variation does occur, it should be revealed by a comparison of toxicity of samples collected over a period of years.

Many residents of the selenium area believe that the cereal grains decrease in toxicity after maturity. Owing to the firmness with which selenium is bound in the protein of cereals (Franke and Painter 1935, 1936), this idea at first appears fatuous. Consideration should be given, however, to the observations of stockmen in the seleniferous area. Some stockmen cling so firmly to their belief that the grains decrease in toxicity that they store the grains for several seasons before feeding them.

Experimental

A summary of results of feeding toxic diets was taken from the records of five years of experimentation. The method of setting up feeding trials has been adequately described in former publications (Franke, 1934, 1934a; Franke and Potter, 1934). The different seleniferous diets fed have different compositions, as shown in Table I.

TABLE I

SELENIFEROUS DIETS FED

	Composition of diets		
	I	II	III
Whole ground grain	70%	82%	82%
Casein	11	10	10
Sucrose	15		
Lard	2		3
Cod-liver oil	x	2	2
Dried yeast	x	2	2
McCollum's salt mixture		4	1
Salt ($NaCl + CaCO_3$)	2		

x = given yeast and cod-liver daily separate from diet.

Although selenium salts have not been added to corn or barley diets, they have been added to several synthetic diets and the conclusion (unpublished) has been reached that there is no difference in toxicity when the different food components vary in amounts no greater than in the diets reported in this paper.

One problem confronting nutrition workers is what responses by the animal best determine the adequacy or inadequacy of a diet. Is it possible to control all the variables so that over a number of years of feeding an absolute value can be assigned each of the several diets used? If this can be accomplished and applied to the diets containing selenium, a direct comparison of the toxicity with the selenium content is at hand.

Before a comparison of the toxicity of different diets can be made, criteria, expressed in mathematical terms, must be chosen upon which to determine relative toxicity. Franke (1934, 1934a), Franke and Potter (1934, 1935), and Franke and Painter (1935) have considered the incidence of death, growth rate, restriction of food consumption, general appearance, gross pathology, and, in some cases, hemoglobin levels, as evidences of the toxicity of a diet. No mathematical comparison is possible with the last three manifestations of selenium poisoning given.

From a toxicological viewpoint, the incidence of death would be the best measure of toxicity. In many cases where the selenium content

was low, no deaths occurred, so death alone can not be taken as the sole measure of toxicity.

The depression of growth by seleniferous feeds is a good criterion of toxicity and it is of practical importance to the stockman. Franke (1935) has studied the effect of small amounts of corn in diets. When toxic corn containing 28 p.p.m. of selenium was 17.5% of a diet, there was depression of growth. The growth rate alone may not show the comparative toxicity of different cereals, or even of like cereals when diets of slightly different composition are used. In nearly all feeding trials a parallel group of control animals was run. There has been some variation in the growth rate of the control groups. The uncertainty of using growth rate of the experimental group can then be partly obviated by taking the difference in growth between the control and experimental animals as a measure of toxicity.

Franke (1934), and Franke and Potter (1935, 1936) have considered the voluntary restriction of food consumption by rats on seleniferous diets a good measure of toxicity. A comparison of toxicity, based on food consumption, should also take into consideration the food consumed by the control group. The average daily food consumption of the control groups on the different diets was not the same. Therefore, the difference between the food consumption of the control and experimental was also used.

For fair interpretation of the results, cognizance must be taken of observations from feeding seleniferous diets. The age of the rats is one evident factor in the effect of seleniferous grains on animals. Franke and Potter (1936a) and Schneider (1936) have found that older animals are much more resistant. Older animals fail to grow satisfactorily, but a few days' difference in age at the time of first feeding selenium has a great effect on the life span.

Females are more susceptible to selenium poisoning than males. The incidence of death from feeding grains and selenium salts is shown in Table II. These data were taken from comparable series in which litter mates and an equal number of males and females were in each group. Although the difference in life span is not great, there is a definite trend.

Nearly all analyses were made on the different lots of grain after part of the lot had been fed. Byers (1935) has shown that samples taken from the same field may vary several-fold in selenium content. This factor was not recognized in the early work, so there is the possibility that the samples were not homogeneous. Such a possibility would probably not cause great error because the samples would ordinarily be fairly well mixed when harvested. In order to overcome this problem, all samples with numbers above 568 were thoroughly

mixed when received. The selenium was determined on air-dried samples by a slight modification of the method described by Robinson, Dudley, Williams, and Byers (1934).

TABLE II

RELATIVE SUSCEPTIBILITY OF MALES AND FEMALES TO SELENIUM POISONING

Series	Source of selenium	Diet number	Rats	Number of deaths during period							
				0–30		30–50		50–70		70–100	
				m.	*f.*	*m.*	*f.*	*m.*	*f.*	*m.*	*f.*
8	No. 388 corn	I	40	3	3	2	3	4	5	6	6
66	No. 582 wheat	II	10	1	4	4	1				
66	No. 583 wheat	II	10	1	1	4	2		2		
94	No. 663 corn	III	10	1	0	1	4	1	0	1	
94	No. 664 corn	III	10					1	2	1	1
62	No. 570 corn	II	10	1	2	2	3	3	1		
33	No. 459 wheat	II	10	0	3	3	1	1	1		
107	Na_2SeO_4	III	10				1	2	2	3	2
107	Na_2SeO_3	III	10	1	4	0	0	2	1	1	1
Total dead by end of period (Grains)				9	15	28	32	37	42	38	44
Total dead by end of period (Se salts)				1	4	1	5	5	8	9	11
Total dead by end of period (All rats)				10	19	29	37	42	50	47	55

The laboratory conditions of the feeding trials varied somewhat during the five-year period. Temperature and humidity are now controlled better than they were in the first experiments.

A summary of the results from the feeding of seleniferous diets is shown in Table III. Only those manifestations of selenium poisoning which could be measured in absolute terms are included in the table. The more factors included in the comparison of toxicity the more accurate will be the results, because there will be less chance for individual differences of animals to alter results and to restrict interpretations.

The measures chosen for comparing toxicity are:

Average life span.
Average net gain.
Difference in net gain (control minus experimental).
Average food consumption.
Difference in food consumption (control minus experimental).

Because all feeding series were not of the same duration, it was necessary to fix the final day of experimentation from which to take the data. The 140th day was chosen as unity for the average life span. Many feeding trials terminated near the end of the 140th day. Nearly all deaths due to selenium poisoning occurred before this day.

The other data were taken during the first 70 days of experimentation. This period is satisfactory because the growth rate of control animals is rapid and apparently is not changed by a few days' difference in age. A longer period might have been chosen but the difference in growth rate is most apparent during the period of rapid growth. When feeding sub-lethal seleniferous diets, growth may be prolonged so that the experimental group approaches the control group in weight after the controls have ceased to grow.

All the data from which the comparison of toxicity was made are given in Table III. The 38 different seleniferous diets are arranged in order of decreasing selenium content. All the data for a given diet are in the row in which the selenium content is given. The age of the sample given in column 3 was the interval of time between maturity of the grain and the date the feeding began. The date of maturity for the small grains was arbitrarily set as July 15, and for the corn, October 15. Time was reckoned from the 15th day of each month.

In columns 11 to 15 inclusive are included the percentages of the experimental animals dead by the 30th, 50th, 70th, 100th, and 140th day of experimentation.

The average net gain (column 17) is the difference between the average of the weights of the experimental animals at the beginning of the series and the average at the end of the 70th day. Where there were deaths, the weight at death was taken as the weight of that animal at the end of its experimental period. In many series there was not the same number of males and females, hence it was necessary to average the males and females separately; otherwise the results would have been in error because males grow much faster than females.

Column 18 gives the difference in gain between the control and experimental groups obtained by the separate average of the males and females. The gain of the control groups for the 70-day periods is the sum of the values in columns 17 and 18.

Column 19 gives the daily food consumption of the experimental groups in grams per rat per day (total grams eaten/the total rat days). The difference in food consumption between the control and experimental animals is shown in column 20. The average daily consumption of selenium is given in column 21. This value, given in gamma, is the product of the average daily food consumption and the p.p.m. of selenium in the diet.

Values of particular interest are shown in columns 22 and 23. The percentages (based on control groups) of food eaten by the rats on seleniferous diets are shown in column 22 and the percentages of gain in weight are shown in column 23. Where there was a net loss in weight the space in this column is left blank.

Columns 24, 25, 26, 27, and 28 give ranks of toxicities obtained by ranking the individual measures of toxicity shown in columns 16, 17, 18, 19 and 20 respectively. The rank of toxicity as determined by each individual measure of toxicity was found in the following manner. The diet which killed the rats in the shortest period (column 16) was considered the most toxic by measure of toxicity *1*. It was therefore ranked *1* in column 24. The diet which killed the rats in the next shortest period would accordingly receive a rank of *2* in column 24. The diet on which the rats gained the least (in many cases there was a net loss) showed that this diet was the most toxic by measure of toxicity *2*, wherefore it received a rank of *1* in column 25. The diet on which there was the greatest difference in gain between the control and experimental rats indicated that it was most toxic by measure of toxicity *3*. It therefore received a rank of *1* in column 26. The diet which caused the greatest voluntary restriction of food intake was the most toxic by measure of toxicity *4*. It received a rank of *1* in column 27. The most toxic diet as measured by the difference in food consumption (measure 5) is the one with the largest value in column 20. This diet would then have a rank of *1* in column 28. These ranks are plotted in Figure 1.

The sums of the five ranks of toxicity for each diet are given in column 29. These sums are probably the most valuable values in the table to determine relative toxicity because they make possible a "summated" comparison of toxicity of diets. By this treatment of the data the diet with the smallest sum (column 29) is the most toxic so it ranked *1* in column 30. In two cases the sum of the five ranks of toxicity was the same. Our basis of comparison indicated the diets equally toxic, so each was given the same rank in column 30. This made it necessary to delete the next number.

In column 31 the diets are ranked from 1 to 38, in order of decreasing selenium content. In the cases where there were two, or three, diets with the same selenium content, they were given the same rank in column 31. When there were two diets with the same selenium content the following number was omitted, and where three diets had the same selenium content the middle number was used. Because there were 38 seleniferous diets to compare, it was desirable to have numbers from 1 to 38 in columns 30 and 31.

It is realized that the deletion of numbers in columns 30 and 31 may introduce a slight error. It would not be expected that the numbers in column 29, which are the sums of the ranks of five bioassay measures of toxicity, would be identical in all cases, even if the toxicity were proportional to the selenium content. Results from animal feeding are rarely that uniform. Suppose diet No. 3 had a

toxic rank of *3* (column 30). There is no number 3 in column 31. The number is 2. By our method of comparison, this diet would be less toxic than if toxicity were directly proportional to the selenium content. Regardless of the inherent error in this method, it is regularly used in statistics and appears to be the best available. Any

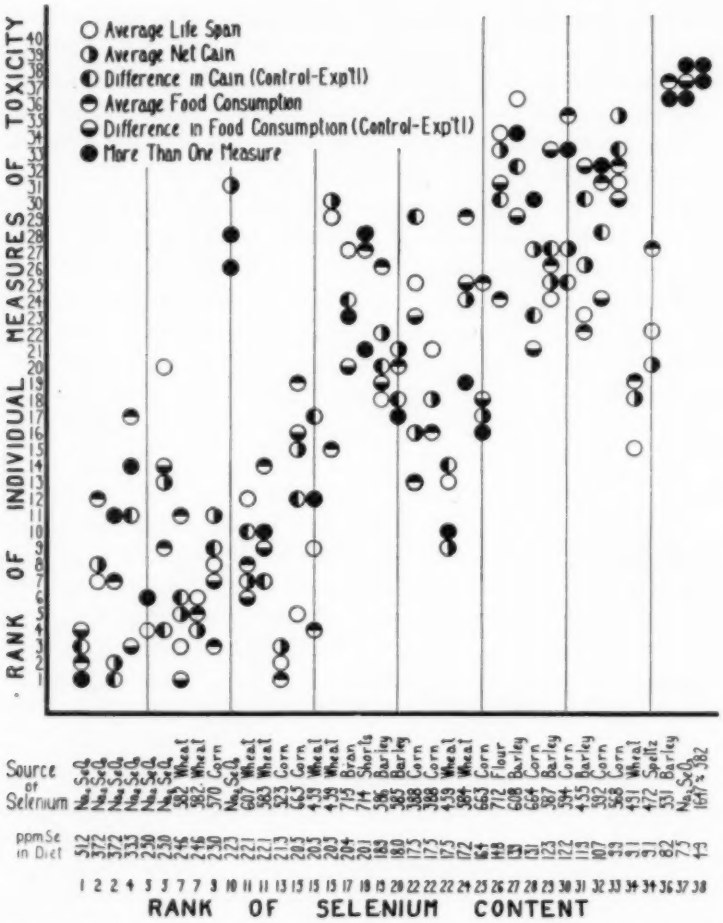

Figure 1. Scatter diagram showing the ranks of the individual measures of toxicity, plotted against the rank of selenium content. A rank of 1 indicates that the diet was found to be the most toxic by the measure of toxicity. The nearer the ranks of toxicity clump to the *X* axis, the more toxic is the diet. "Speltz" should be replaced by "emmer" in Figure 1 and Table III.

errors due to this method of comparison would be small. In no case would the difference between the values in the same rows in columns 30 and 31, due to our method of ranking, be greater than $\pm$ 1.

In column 32, *d*, which is the difference between the values in columns 31 and 30, shows the degree of relationship between the

selenium content and toxicity. If d is negative, the toxicity is less, and if positive, the toxicity is more than it would be if toxicity and selenium content were strictly proportional.

In eight cases there were no control groups, hence no values for columns 18 and 20 could be obtained from the feeding records. In order to use these series in the comparison of toxicity of the diets, arbitrary ranks were given in columns 26 and 28. The ranks given in these columns are the same as those in column 31. These are the ranks that would be in these columns if these two measures of toxicity (difference in growth and food consumption between the control and experimental) were in direct proportion to the selenium content in all

TABLE III

SUMMARY OF FEEDING SELENIFEROUS DIETS

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Diet Number	Selenium content p.p.m.*	Source of Selenium	Age of sample	Series Number	Date of series	Diet fed	Number of rats		Average age in days		Per cent dead by day of experimentation				
			Yr. Mo.				M	F	M	F	30	50	70	100	140
1	51.2	Na_2SeO_3†		97	8- 1-34	III	5	4	28	29.5	100				
2	37.2	Na_2SeO_3		96	12-12-33	III	3	5	29	29	37.5	87.5	100		
3	37.2	Na_2SeO_3		107	24- 9-34	III	5	5	29	28	50	50	80	100	
4	33.5	Na_2SeO_3		97	8- 1-34	III	5	4	28	29	55.6	66.7	86.7	77.8	88.9
5	25.0	Na_2SeO_4		96	12-12-33	III	3	5	29	29	50	100			
6	25.0	Na_2SeO_4		107	24- 9-34	III	5	5	29	29	0	10	50	100	
7	24.6	582 Wheat	1	66	18- 8-32	II	5	5	29	29	50	100			
8	24.6	582 Wheat	9	85	4- 5-33	II	5	5	30	31	40	90	100		
9	23.0	570 Corn	8	62	26- 6-32	II	5	5	29	29	30	90	100		
10	22.3	Na_2SeO_3		97	8- 1-34	III	5	4	28	29	0	0	22.2	33.3	33.3
11	22.1	607 Wheat	6	81	8- 2-33	II	6	4	30	30	40	60	80	100	
12	22.1	583 Wheat	1	66	18- 8-32	II	5	5	28	28	20	80	100		
13	21.3	523 Corn	1	40	4-11-31	II	2	3			60	100			
14	20.5	665 Corn	1	94	14-11-33	III	5	5	29	29	40	90	100		
15	20.5	459 Wheat	1-6	47	30- 1-32	II	6	4	29	28	20	100			
16	20.5	459 Wheat	4-9	121	2- 5-35	III	4	6	28.5	29.8	0	10	30	40	60
17	20.4	715 Bran‡	4-8	119	27- 3-35	spe.	4	6	28.5	28.3	0	10	30	50	70
18	20.1	714 Shorts‡	4-9	121	2- 5-35	spe.	4	6	28.5	29.8	0	20	30	40	60
19	18.9	586 Barley	1	68	9- 9-32	II	4	3	29	29	0	14.3	42.9	100	
20	18.0	585 Barley	1	68	9- 9-32	II	4	3	29	29	0	28.6	57.1	100	
21	17.5	388 Corn	2-1	1	11-12-29	I	6	9	30.3	30	0	6.7	40	73.3	100
22	17.5	388 Corn	3	8	1-11-30	I	20	20	29.0	29	15	27.5	50	80	100
23	17.5	459 Wheat	3	9	6-11-30	I	6	9	29	28	40	46.7	80	100	
24	17.2	584 Wheat	1	66	18- 8-32	II	4	6	28	28	0	40	70	80	90
25	16.4	663 Corn	1	94	14-11-33	III	5	5	29	29	0	60	70	80	90
26	14.8	712 Flour‡	4-8	119	27- 3-35	III	4	6	29.2	28.3	0	0	0	0	20
27	13.9	608 Barley	3	80	16- 1-33	II	2	3	28	28	0	0	0	0	0
28	13.1	664 Corn	1	94	14-11-33	III	5	5	29	29	0	0	30	50	50
29	12.3	587 Barley	1	68	9- 9-32	II	4	3	29	29	0	28.6	42.9	71.4	71.4
30	12.2	594 Corn	10	88	18- 8-33	III	4	6	29	29	0	10	10	20	30
31	11.9	455 Barley	5	12	15-12-30	I	9	6	28	28	0	13.3	46.7	67.0	86.7
32	10.7	592 Corn	1-5	84	13- 4-33	II	6	2	30	31	0	12.5	25	37.5	50
33	9.9	568 Corn	8	62	26- 6-32	II	5	5	29	29	0	20	40	40	40
34	9.1	471 Wheat	4	10	3-12-30	I	2	3	28.5	28	0	0	100		
35	9.1	472 Speltz	4	10	3-12-30	I	3	2	28.7	28	0	20	60	60	100
36	8.2	531 Barley	4	41	20-11-31	II	2	3	28.0	28.7	0	0	0	0	0
37	7.5	Na_2SeO_3		103	24- 7-34	III	5	5	29	29	0	0	0	0	0
38	4.9	16.4% 582	2	103a	27- 7-34	III	5	5	28	29	0	0	0	0	0

* Probable error of selenium content of grains = ± 1.32 p.p.m. selenium.
† All selenium salts were added to control wheat diet No. III.
‡ Milled from 459 wheat.

TABLE III—*Continued*

SUMMARY OF FEEDING SELENIFEROUS DIETS

16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	
Individual measures of toxicity								Statistical study									
Average life span, days	Average net gain, grams	Diff. in gain grams control-exptl.	Food eaten exptl. gram per rat day	Diff. in food control-exptl.	Se Consumed gamma Per rat day	Food eaten by exptl. exptl./control ×100	Weight gained exptl./control ×100	Rank of individual measures of toxicity					Sum of ranks	Toxic rank of diets	Se rank of diets	Difference in rank "d" (column 31–30)	Diet Number
1	2	3	4	5				1	2	3	4	5					
13.1	−23.6	205.1	2.75	9.87	140.8	21.8		1	1	3	2	4	11	1	1	0	1
34.3	−5.1		4.51		166.8			7	8	2	12	2	31	5	2	−3	2
44.1	−18.7	223.1	3.76	9.49	139.9	28.4		11	2	1	7	11	32	6	2	−4	3
54.2	3.7	177.8	5.73	6.89	213.2	45.0	2.0	14	14	11	17	3	59	13	4	−9	4
32.9	−7.7		3.69		92.3			4	6	5	6	5	26	2	5	+3	5
68.9	1.3	203.1	4.18	9.07	104.5	31.5		20	13	4	9	14	60	14	5	−9	6
29.1	−11.0	191.6	4.21	10.09	103.6	29.4		3	5	6	11	1	26	2	7	+5	7
33.6	−16.0		3.55		87.3	25.0		6	4	7	5	7	29	4	7	+3	8
34.4	−4.0	179.1	2.98	9.68	68.5	23.5		8	11	9	3	7	38	8	9	+1	9
91.7	71.8	109.7	7.82	4.80	174.4	62.0	39.6	26	31	25	28	28	139	30	10	−20	10
44.7	−7.2	178.7	3.88	9.86	85.7	28.2		12	7	10	8	6	43	9	11	+2	11
40.9	−4.5	185.1	4.63	9.67	102.3	32.4		10	10	7	14	9	50	10	11	+1	12
25.2	−16.5		2.69		57.3			2	3	13	1	13	32	6	13	+7	13
33.4	14.1	167.7	6.00	8.43	123.0	41.6	7.7	5	15	12	19	16	67	15	15	0	14
38.4	0.5	147.2	3.54	9.39	72.3	27.4	.03	9	12	17	4	12	54	11	15	+4	15
102.3	63.4		5.28		108.2			29	30	15	15	15	104	19	15	−4	16
96.9	54.7	115.3	6.39	6.59	130.4	49.2	32.2	27	23	24	23	20	117	23	17	−6	17
99.3	62.7	119.0	6.23	5.01	125.2	55.4	34.5	28	26	21	21	27	125	25	18	−7	18
67.9	47.1	119.7	6.99	7.01	132.1	49.9	28.2	18	22	20	26	19	105	20	19	−1	19
63.9	36.4	130.4	6.01	7.99	108.2	42.9	21.8	17	20	18	20	17	93	17	20	+3	20
82.9	15.3	96.3	4.55	5.74	79.6	44.2	13.2	25	16	29	13	23	106	21	22	+1	21
71.0	24.3		5.69		100.1			21	18	22	16	22	99	18	22	+4	22
49.1	−4.9	162.1	4.19	9.51	73.3	30.6		13	9	14	10	10	56	12	22	+10	23
68.6	54.8	125.8	8.06	5.24	138.6	56.4	30.3	19	24	19	29	25	116	22	24	+2	24
62.4	21.2	160.6	6.66	7.77	109.2	46.2	11.7	16	17	16	25	18	92	16	25	+9	25
138.1	96.4	85.3	6.59	3.65	97.5	64.4	52.0	34	33	30	24	31	152	32	26	−6	26
140	109.3	68.2	9.33	4.27	129.7	68.6	61.6	37	34	32	34	29	166	35	27	−8	27
103.4	62.9	118.7	8.68	5.75	113.7	60.2	34.6	30	27	23	30	21	131	26	28	+2	28
82.1	58.0	108.8	8.95	5.04	110.2	64.0	34.8	24	25	27	33	26	135	28	29	+1	29
122.0	61.6	114.2	10.13	2.91	123.6	77.7	35.0	33	27	25	35	33	153	33	30	−3	30
82.0	61.4	85.3	6.35	3.30	75.6	63.8	41.9	23	26	30	22	32	133	27	31	+4	31
110.4	72.0	98.4	8.72	5.57	93.3	61.0	42.3	32	32	28	31	24	147	31	32	+1	32
104.6	116.7	58.4	8.90	3.76	95.2	70.3	66.6	31	35	33	32	30	161	34	33	−1	33
57.6	27.4		5.90		53.7			15	19	34	18	34	120	24	34	+10	34
79.8	28.6		7.28		65.5			22	30	34	27	34	137	29	34	+5	35
140	127.8	14.8	11.46	0.55	94.0	94.6	89.6	37	36	36	37	36	182	36	36	0	36
140	175.1	6.2	11.18	0.01	83.9	100.0	96.6	37	38	38	36	37	186	37	37	0	37
140	173.1	8.2	11.59	−0.40	56.8	103.6	95.5	37	37	37	38	38	187	38	38	0	38

* Probable error of selenium content of grains = ± 1.32 p.p.m. selenium.
† All selenium salts were added to control wheat diet No. III.
‡ Milled from 459 wheat.

cases. It is realized that this method of assigning arbitrary ranks tends to exaggerate the relationship between the toxicity of the diets and selenium content. There will be a tendency to decrease the magnitude of the numbers in column 32.

Statistical Analysis of Data

In most cases the reader can readily interpret biological data from tables or charts. In this case, however, we feel that the data in Table III warrant further analysis to bring out significant facts, inasmuch as the values given are summaries of the results of a group of experimental animals.

Several statistical methods may be used to show the relationship between the variables in Table III. Among those used, the coefficient of correlation, r, is probably the best single means of showing covariance. It should be kept in mind that a correlation coefficient of .90 is nearly three times as certain as when $r = .60$.

Two methods of obtaining r appear applicable to the data in Table III. One determines the relationship of the variables by their rank. In this case the following equation was used:

$$\rho_{xy} = 1 - \frac{6\Sigma(X - Y)^2}{n(n^2 - 1)} \tag{1}$$

where ρ_{xy} = correlation coefficient between X and Y,
X = rank in selenium content,
Y = rank of toxicity,
$X - Y = d$(column 32, Table III),
Σ = sum.

The other method tells the relationship between the toxic effects and selenium content. In this case the useful form of the Bravais-Pearson coefficient of correlation (Richardson, 1935) was used:

$$r_{xy} = \frac{n\Sigma XY - \Sigma X \Sigma Y}{\sqrt{n\Sigma X^2 - (\Sigma X)^2}\sqrt{n\Sigma Y^2 - (\Sigma Y)^2}}, \tag{2}$$

where r_{xy} = correlation coefficient between X and Y,
X = selenium content,
Y = a measure of toxicity,
n = number of cases.

Correlation coefficients were determined for each of the measures of toxicity by formulas 1 and 2. All the data necessary for the calculation of the correlation coefficients are in Table III. The selenium content (X values) is in column 1, the rank in column 31. The column from which the measures and ranks of toxicity (Y values) were taken is given after the correlation coefficient.

Measure of toxicity	r_{xy}	Column of Y value	ρ_{xy} [1]	Column of Y value
1. Average life span	$-.69 \pm .06$	16	$.75 \pm .05$	24
2. Average net gain	$-.74 \pm .05$	17	$.78 \pm .04$	25
3. Difference in net gain (control minus experimental)	$.79 \pm .05$	18	$.86 \pm .03$	26
4. Average food consumption	$-.70 \pm .06$	19	$.80 \pm .04$	27
5. Difference in food consumption (control minus experimental)	$.70 \pm .06$	20	$.79 \pm .05$	28
Rank of Σ of ranks			$.86 \pm .03$	30
Σ of ranks	$-.80 \pm .04$	29		

[1] The arbitrary ranks given to measures of toxicity 3 and 5 were not included in the calculations for the individual measures of toxicity, neither are they included in Figure 1.

One may ask if it was necessary to use several measures of toxicity, when one may suffice. A scatter diagram of the ranks of the individual measures of toxicity (Figure 1) shows that there are wide variations in the individual measures, but that there is a definite trend. In some cases the ranks of the individual measures of toxicity of a specific sample are grouped, while in other cases they show that there is a great difference in the general response of groups of animals to the different seleniferous diets. No consistent arrangements in rank of the different measures of toxicity can be found for any group of diets containing selenium from the same source.

From a statistical consideration the two correlation coefficients, $r_{xy} = .80$, and $\rho_{xy} = .86$, for all diets, indicate a high degree of correlation between the toxicity and selenium content. Due to the number of cases, the method of setting up comparable feeding experiments, and the method of determining relative toxicity of diets, the authors believe a higher degree of relationship should exist between the selenium content and toxicity of the diets, if the selenium were equally poisonous in each of the sources of selenium.

There are several groups of diets shown in Table III containing selenium from the same source. If there is a difference in the toxicity of selenium from the different sources, further analysis of the data in Table III should demonstrate it.

The coefficients of correlation using both formulas 1 and 2 were determined for the group of diets containing sodium selenite, wheat, corn, and barley. When computing ρ_{xy} it was necessary to rerank the individual groups according to selenium content and toxicity. In computing r_{xy} the selenium content was taken as the abscissa (X) and the sum of the individual ranks of toxicity as ordinate (Y).

Number of diets	Number of experimental animals	Source of selenium	ρ_{xy}	r_{xy}
6	55	Sodium selenite	.97 ± .02	.96 ± .02
9	90	Wheat [1]	.98 ± .01	.95 ± .02
10	128	Corn	.93 ± .03	.97 ± .01
6	46	Barley	.72 ± .13	.86 ± .07
38	382	All diets	.86 ± .03	.80 ± .04

[1] Wheat diet in column 16 was not included.

It has now been shown that there is a higher degree of correlation between the toxicity of the diets containing selenium from the same source than there is between the toxicity and selenium content of all the diets. The selenium in some of the diets must be in a more toxic form than in other diets, otherwise the correlation coefficients for the

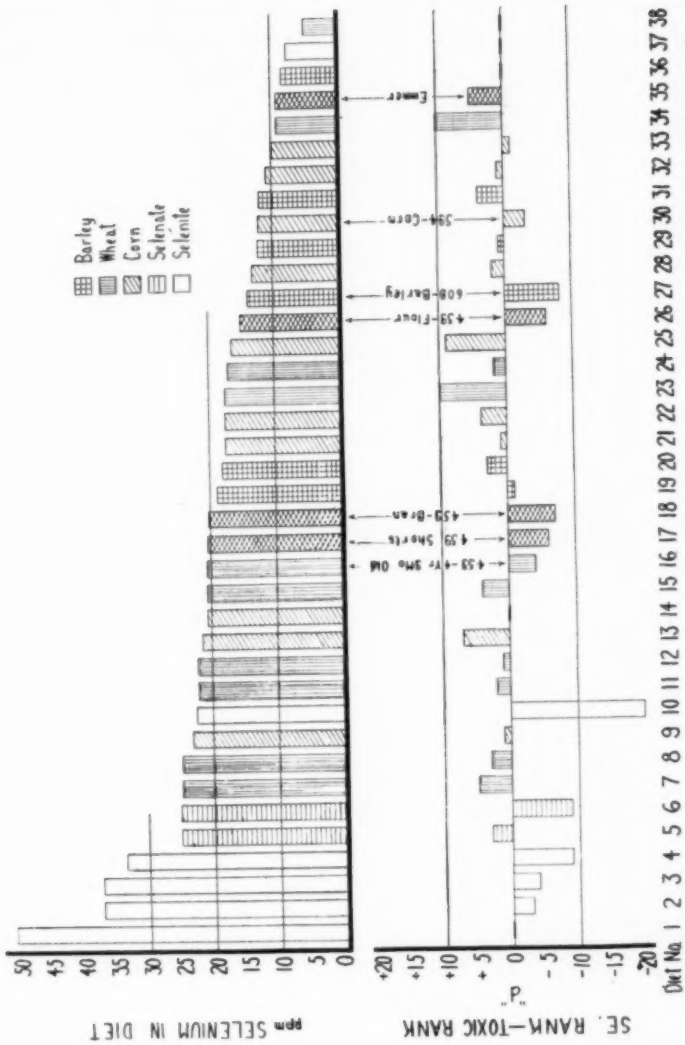

Figure 2. The difference in rank (column 32, Table III) is shown below the selenium content of the diet. If the rank of toxicity and rank of selenium content were the same, *i.e.*, $d = 0$, all would fall on the line at zero. The sign of d indicates whether or not the diet is more or less toxic than it would be if the selenium were found to be equally toxic in all diets.

entire group of diets would have approached r for the group of diets containing selenite, corn, or wheat.

Figure 2 shows the difference in rank between the X values (selenium content) and Y values (sum of ranks of toxicity) of the diets in Table III. Diets of special interest are indicated but they will not be discussed here. The figure shows that there is some consistency in the sign and magnitude of a d for the diets containing selenium from the same source.

The differences in rank shown in Figure 2 should be of value to determine which group of diets contains selenium in the most toxic form. The sum of the differences, d (column 32, Table III), for each group of diets, was divided by the number of diets, n. The order of toxicity of selenium from the five sources is indicated in Table IV by the sign and size of the average value for each group of diets. The larger positive value indicates more toxic selenium.

TABLE IV

TOXICITY OF SELENIUM FROM DIFFERENT SOURCES

Source of selenium	Number of diets n	Number of rats	Sum of d (column 32, Table III)	$\Sigma d/n$	Order of toxicity of selenium
Wheat	9	90	+37	+4.11	1
Corn	10	128	+21	+2.10	2
Barley	6	46	− 3.0	−0.50	3
Na_2SeO_4	2	18	− 6.0	−3.0	4
Na_2SeO_3	6	55	−36.0	−6.0	5

In the calculation of the correlation coefficient r_{xy}, it was assumed that a linear relationship existed between the sum of the individual ranks of the measures of toxicity and the selenium content. The larger correlation coefficients were adequate proof of this relationship. The lines of regression of Y on X for each of four groups of diets are plotted in Figure 3. The best fitting line for each group of points was determined by the method of least squares.

In four cases there were two diets containing selenium at the same level from the same source. An inspection of these should indicate if our method of determining relative toxicity is dependable. The sums of the individual ranks of toxicity for each pair of diets containing selenium from the same source at the same level were: for selenite 31 and 32, for No. 582 wheat 26 and 29, and for No. 388 corn 99 and 106. These show good agreement. The sums of the ranks of toxicity for the diets containing 25 p.p.m. of selenium as sodium selenate were 26 and 60. Reference has been made to the apparent difference in toxicity of different lots of a selenium salt (Franke and Potter, 1935).

It should be recalled that the feedings of these diets containing selenium from the same source were not carried out at the same time.

The results of biological experiments are usually dependent upon a number of variables working together or independently. If we can determine to what degree other factors than selenium affected the results, it should be possible to discover if selenium is the limiting factor.

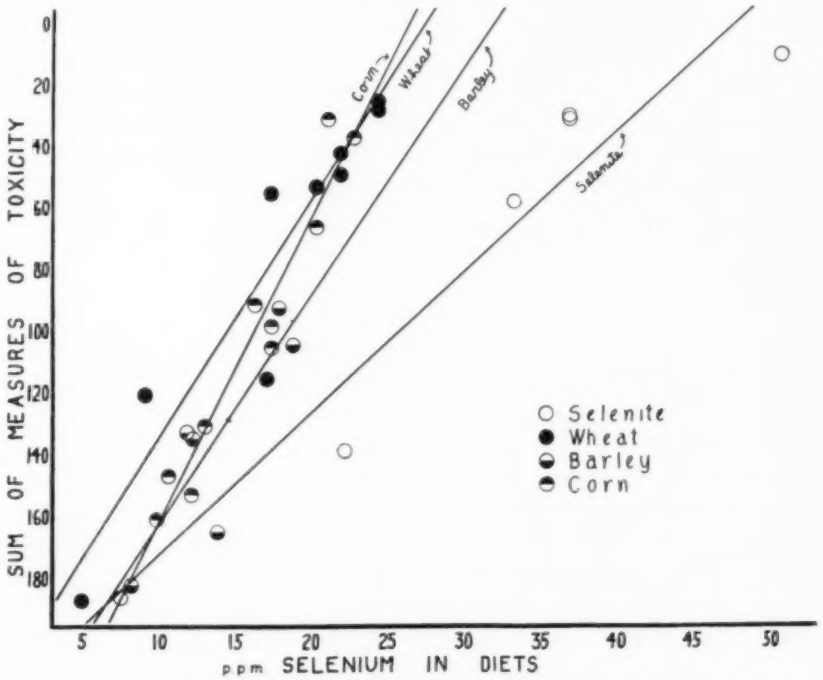

Figure 3. Regression lines showing the relative toxicity and increase in toxicity with an increase in selenium content of four groups of diets. The smaller the sum of the measures of toxicity, the more toxic is the diet.

The correlation coefficient for measure of toxicity (3) (average net gain) of .74 indicates that other variables than the difference in toxicity of selenium from the different sources reduced the magnitude of r. None of the correlation coefficients for the groups of diets containing selenium from the same source reached unity. The problem is to determine to what degree the variability of experimental conditions reduced the correlation coefficients.

The standard error of estimate, a measure of the dispersion about the regression line, is given by the equation:

$$(3) \qquad S_y = \sqrt{\frac{\Sigma(y - \bar{y})^2}{n}},$$

where y = observed values,
$\bar{y}$ = computed values (or regression line),
n = number of observations.

Any regression line in Figure 3 shows the dispersion of the observed values about the regression line. In the present case the units measured on the ordinate (Y axis) would be in grams instead of the sums of the ranks of toxicity as shown in Figure 3. The standard error of estimate is a measure of the dispersion due to all other variables because when $S_y = 0$, all the points fall on the regression line and $r = 1$.

The standard deviation, σ_y, a measure of the total dispersion on the Y axis, is given by the equation:

$$\sigma_y = \sqrt{\frac{\Sigma(y - \bar{y})^2}{n}}, \tag{4}$$

where y = observed value,
$\bar{y}$ = mean of y.

The standard deviation is made up of two components:

a. The dispersion due to difference in selenium content of the diets.
b. The dispersion due to all other variables.

The control groups did not grow at the same rate as shown by the standard deviation of 22.4. If the experimental animals were subject to the same variation as the control animals, this variable should cause a dispersion about the regression line, S_y, equal to the dispersion on the Y axis. From the relation

$$S_y = \sqrt{S_{y \cdot 0}^2 - \sigma_{y \cdot c}^2}, \tag{5}$$

where $S_{y \cdot 0}$ = observed standard error of estimate,
$\sigma_{y \cdot c}$ = standard deviation of control groups,

we can determine how much closer the observed values would come to the regression line had there been no variability in experimental conditions. By equation (3) $S_{y \cdot 0} = 32.3$ and by equation (4) $\sigma_{y \cdot c} = 22.4$. These values substituted in equation (5) gave $S_y = 23.3$. If there had been no variability of experimental conditions, the standard error of estimate would have been 23.3 instead of 32.3. This value, $S_y = 23.3$, can be substituted in an equation for r_{xy}:

$$r_{xy} = \sqrt{1 - \frac{S_y^2}{\sigma_y^2}}. \tag{6}$$

where σ_y = standard deviation of experimental groups.

From equation 6, r_{xy} was found to be .88. If we are justified in making the assumption that the experimental groups were subject to the same variation as the control groups, we can say that the correlation coefficients would have been .88 instead of .74.

The prediction value of a correlation coefficient (probability of any value falling within a given distance from the regression line) is given by the relation, $1 - \sqrt{1 - r^2xy}$ (Treloar, 1935). When the value of r increased from .74 to .88, the prediction value increased by 1.6.

It does not seem amiss to assume that the correlation coefficients would have higher values if it were not for the factors inherent in the experimental conditions. When the prediction value is increased 1.6 times, the correlation coefficients would approach unity for some of the diets containing selenium from the same source. When the prediction value of a correlation coefficient of .95 is increased 1.6 times, the correlation coefficient becomes approximately .98. It can be seen that some of the correlation coefficients would exceed .99.

Discussion

From the correlation coefficients for the five measures of toxicity it is most significant that for the large number of cases which are included in Table III, each shows nearly the same degree of correlation. With individual samples single measurements may vary within wide limits as Figure 1 shows. This emphasizes the necessity for several measures of toxicity to describe adequately a given sample but for the entire group of diets any single measure appears to show nearly the same degree of relationship between toxicity and selenium content.

The correlation coefficients found from the ranks of the measures of toxicity, ρ_{xy}, are slightly higher than those obtained from the recorded values obtained from the records of feeding trials, r_{xy}, probably because there is not a uniform change in the selenium content of the diets. The first four diets have high selenium contents (Figure 2) which rank does not take into account, but the toxicity of these diets is near that of some of the cereal diets.

The values of the correlation coefficients for the measure of toxicity, average life span, are lower than for the other measures of toxicity, because it was necessary to fix the final day of experimentation. All feeding trials did not continue until all animals died. There were four diets which did not kill a single rat so this measure of toxicity must record them equally toxic. The other measures of toxicity show that these four diets were not equally toxic.

That the diets containing selenium from the same source (selenate is an exception) are consistent in their rank is apparent from Figure 2.

There is not a diet containing sodium selenite with a positive sign for the value of d. The first diet (Table III) contained so much more selenium than any of the other diets (51.2 p.p.m.) that it would be expected to be the most toxic. As the selenite diets approach a selenium content comparable to the cereal diets the magnitude of d uniformly increases. The value of d for the selenite diet number 37 is 0, but the next diet containing a toxic cereal contains only two-thirds as much selenium. The two diets have approximately identical toxicities because the sums of the individual ranks of toxicity are 186 and 187.

Only one wheat diet has a negative value for d. The exception is diet number 16, in which the sample was nearly five years old when fed. All other toxic wheat diets have positive values from 1 to 10 for d except diet No. 38, in which $d = 0$. In not a single case is the sum of the five ranks of toxicity for a given wheat diet less than the sum for a wheat diet containing more selenium. This is also true of the diets containing sodium selenite.

The corn and barley diets likewise show good agreement within their groups. Only two of the ten corn diets have negative values for d (Figure 2). In three cases the toxicity was not in the same order as the selenium content (Table III) but the difference in selenium content was of about the same order of magnitude as the probable error in selenium analysis, and the sums of the individual ranks of toxicity differed only slightly. Only one barley sample (No. 608) is out of line with the rest of the barley group.

The remarkably high correlation in the groups of diets containing selenium from the same source indicates that the selenium in each kind of grain has a degree of toxicity which is specific for each kind of cereal. The toxicity of selenium in the different groups in Table IV was found to be: wheat > corn > barley > selenate > selenite.

The correlation coefficients for the corn, wheat and selenite diets suggest a perfect causal relationship between selenium content and toxicity. If we assume that there was the same variability of experimental groups as there was of control groups, and use the device of increasing the prediction value, some values of r become so near unity as to indicate that selenium is the limiting factor. The values of r for the wheat and corn diets are approximately the same as for the selenite diets in which selenium is known to be the sole toxicant. This is evidence against the presence of another toxicant, unless it is directly associated with selenium. *Until the presence of other poisons is demonstrated, it can be assumed that the toxic effects produced in experimental animals are due to selenium.* Other poisons have not been found and those suggested as possibilities were not as toxic as selenium by oral feeding (Franke and Moxon, 1937).

Table IV further shows that there is little difference in toxicity of selenium in the different cereals. The value of $\Sigma d/n$ for the group of barley diets would have been 1.16 had it not been for the diet containing barley No. 608. The position of the barley group, however, would not have been changed.

The lines of regression, Figure 3, plainly show the relative toxicity of the different groups of diets. From the point of intersection of any horizontal line with each of the regression lines one should be able to determine the selenium content necessary to have four equally toxic diets from each of the four sources. Suppose a horizontal line were drawn from the point where the sum of the ranks of toxicity is 105. The wheat diets containing 14 p.p.m. of selenium, the corn 16 p.p.m., the barley 18 p.p.m., and the selenite 25 p.p.m. would have equal toxicities. The regression lines also show the relative increase of toxicity with selenium content. The points fit the curves fairly well. The curves show that there is less difference between the toxicity of selenium in the different cereals than between the cereals and selenite.

The results are not conclusive proof that selenium in wheat is more toxic than selenium in corn or barley as is shown in Table IV. Possibly a similiar slight difference in observed toxicity would have resulted if selenium salts had been added to a barley or corn diet. Our feeding trials indicate on the other hand that in such cases a difference in observed toxicity would not be probable. The correlation coefficient ρ_{xy} for all diets containing the cereals grown in 1932 is + .94. These included five wheat, six corn, and four barley diets. The coefficient .94 shows about as high a degree of relationship as was found for the diets containing selenium from the same source. It has been shown (Painter and Franke, 1935) that the selenium in the cereals is in organic compounds. It is doubtful if the cereals would synthesize different compounds of selenium; however, the relative proportion of the compounds may vary in the different grains.

The slope of the regression line for the corn diets is greater than the slope for the other cereal diets (Figure 3). With increased selenium content in the corn diets there is a more rapid increase in toxicity. The curves for wheat and corn intersect at about 23.0 p.p.m. The curves indicate that above this level corn diets would be more toxic than wheat diets containing equal amounts of selenium. Three corn diets with selenium contents near the extreme levels have greatly affected the slope of the curve.

Only eight of the thirty diets containing selenium as it occurs in seed grains have negative values in column 32, Table III, or Figure 2. Two of these are diets containing corn sample No. 594 and barley sample No. 608. These appear to have the least toxic selenium in their

respective kinds of grain. These two samples were grown the same year in the same field. The other sample grown in this field at the same time, No. 607 wheat, shows a toxicity consistent with the other wheat samples.

Four of the cereal diets of low toxicity contain wheat No. 459 and its milled fractions. Sample No. 459 was fed several different times. The results are given in tabular form to show the apparent change in toxicity. It is probable that the value of *d* for diet No. 16 would have been a larger negative number had not arbitrary values been given ranks of measures of toxicity 3 and 5. It is evident that this sample showed a decrease in toxicity as it aged.

Diet number	Sample number	Age of sample when fed	Value of *d* [2]
23	459 Wheat	3 months	+10
15	" "	1 year, 6 months	+4
16	" "	4 years, 9 months	−4
26	712 Flour [1]	4 years, 8 months	−6
17	714 Bran [1]	4 years, 8 months	−6
18	715 Shorts [1]	4 years, 9 months	−7

[1] Milled from 459 wheat a short time before fed.
[2] Average value of *d* for wheat = + 4.11.

The results with the milled products show that the selenium in the bran, flour and shorts is equally toxic. Horn, Nelson and Jones (1936) found that the "toxic principle" was quite uniformly distributed in the milled fractions of wheat.

Franke and Potter (1935) suggest that some of the selenium in selenite may be reduced to metallic form before it is absorbed. They found metallic selenium non-toxic and selenide slightly toxic. Selenates are much more difficult to reduce than selenites and selenides oxidize to metallic selenium readily in air. The toxicity of selenium from all the sources fed is in the order: wheat > corn > barley > selenate > selenite > selenide > metallic selenium. Based on the degree of inhibition of CO_2 production by yeast cells, Moxon and Franke (1935) found the above order of toxicity of selenate and selenite reversed. The selenite was very toxic and the selenate virtually non-toxic. From injecting selenium salts into albino rats, Franke and Moxon (1936) found the minimal fatal dose for selenite less than for selenate. The mechanism of selenium toxicity in all its forms can hardly be attributed to its reduction. It is probable that selenium as it occurs naturally in cereals is not in a high state of oxidation.

The amount of selenium consumed in gamma per rat per day, shown in column 21, does not appear to correlate with the other data. In some cases relatively large quantities of selenium were consumed

without causing a large percentage of deaths in the animals. The rats on the diets containing selenium salts ate more selenium than those fed selenium in the toxic grains. It might be suspected that the toxic symptoms observed in rats would be a direct function of the quantity of selenium consumed per day. By ranking the values in column 21 and determining the difference between this rank and the rank of toxicity of the diets no relationship was found between the selenium consumed and the observed toxicity. The correlation coefficient ρ_{xy} as found by formula 1 had a value of .03 which proves that virtually no relationship exists. Rats on diets containing 10 to 15 p.p.m. of naturally occurring selenium ate more selenium per day than the rats on diets containing about 25 p.p.m. of naturally occurring selenium. Although there is a high correlation between the selenium content of the diet and voluntary food restriction, there is no correlation between the toxic effects produced and the daily consumption of selenium. In selenium poisoning there is a pronounced restriction of food consumption which is not great enough to cause death by starvation in control rats (Franke and Potter, 1935) but the food intake is reduced to a level which will not maintain life and permit the animal to withstand the poisonous action of selenium. In general, when there was a tendency toward voluntary food restriction, the rats eating the most selenium fared the best. This can be partly explained by the fact that those animals more resistant to selenium poisoning ate more as they became older. Some rats on lower levels of selenium grow so they ingest larger quantities of selenium as their body weights increase.

It has been shown (Franke, 1934; Franke and Potter, 1935, 1936) that animals fed seleniferous diets *ad lib.* voluntarily restrict their food consumption. The actual food consumption (based on percentage of control) is shown for the seleniferous diets in column 22. In all cases excepting the two diets with the lowest selenium content, the experimental rats ate less than the control rats. When the diets contained more than 10 p.p.m. of selenium, the food consumption was reduced approximately 30%. With some diets containing extremely toxic concentrations, the restriction of food reached nearly 80%.

It may be believed that the restriction of food consumption is entirely responsible for the retarded growth rate of animals fed diets containing small amounts of selenium. The values in column 23 show that in every case the percentage of gain of the experimental animals is less than the percentage eaten. There can be no question that the retarded growth caused by small amounts of selenium is not entirely due to reduced food intake. Since animals receiving seleniferous diets voluntarily restrict their food consumption, a greater proportion of the total food consumed is necessary for maintenance. No doubt this is

partly responsible for the observed results. The fact that the animals on a seleniferous diet gain less per gram of consumed food is of tremendous economic importance to the farmer feeding seleniferous grains. He cannot obtain the net unit gain that he could if he fed a "normal" grain.

The minimum concentration of selenium that may be harmful has not yet been found. It is safe to predict that any diet containing over 5 p.p.m. of selenium will retard the growth of young animals. Relatively small quantities of selenium in the diet will produce death. All diets but one shown in Table III containing more than 9.1 p.p.m. of selenium caused deaths in young animals. These conclusions are based on studies on the rat. Recently Smith, Stohlman, and Lillie (1937) have shown the rat to be more resistant to selenium poisoning than the cat or rabbit. Little is known about the relative susceptibility of farm animals to the poisonous action of selenium.

Summary and Conclusions

The results of feeding 38 seleniferous diets to albino rats have been summarized. Three hundred and eighty-two experimental animals were used in these feeding trials. In most of the trials the experimental group was paralleled by a control group. The sources of selenium were sodium selenite, sodium selenate and naturally occurring selenium in wheat, corn, barley and emmer. The rank of toxicity and relative toxicity of the diets have been determined and the toxicity compared with the selenium content.

Correlation coefficients were computed for all diets, and for the diet groups containing selenium from the same source. A high degree of correlation was found between the toxicity and selenium content. The correlation coefficients for the groups of diets containing selenium from the same source were usually larger than for the entire group of diets. It was shown that the selenium from the different sources varied in toxicity. If selenium is the sole toxicant in the cereals fed, the relative toxicity of selenium in the different diets is in the order: wheat > corn > barley > selenate > selenite.

If the experimental animals were subject to the same variability as the control animals, and this is taken into account in the calculation of the correlation coefficients, some values of r become so near unity that it can be assumed that selenium is the limiting factor.

There was a pronounced restriction of food consumption in every diet containing over 10 p.p.m. of selenium as it occurs in the cereals.

The percentage of gain in the experimental animals was less than the percentage of food consumption (percentage based on control

groups). This means that the gain per gram of diet consumed was less for the seleniferous diets than for control diets.

Concentrations of less than 5 p.p.m. of selenium in diets will prevent normal growth. In every case shown in Table III the rats on seleniferous diets gained less than the control rats.

All but one of 35 diets containing over 9.0 p.p.m. of selenium caused deaths in young animals. It is probable that only naturally occurring selenium will regularly cause deaths at this level.

Female rats are slightly more susceptible to selenium poisoning than males.

There was no relationship between the absolute amount of selenium consumed per day and observed toxic effects. The effect of seleniferous diets appears to depend more on the concentration of selenium in the diet than upon the quantity of selenium consumed per day.

Evidence is presented that a toxic grain decreased in toxicity after it had been stored several years.

If the results obtained by feeding rats can be applied to larger animals, it is evident that farmers have suffered great losses by feeding seleniferous feeds with surprisingly low selenium contents.

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IMBIBITION OF WATER BY PROTEINS

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(Read at the Annual Meeting, May 1937)

Proteins, as they occur in nature, always occur in association with water. Dry proteins are purely artificial products and special precautions have to be taken in their preparation. Moreover, the dividing line between a protein which has lost water till it is dry and one which has lost water till it has begun to change its character is not always very well defined.

Proteins are hygroscopic bodies and dry proteins placed in contact with water as vapor or as liqud absorb it with evolution of heat and loss of total volume. The amount of water absorbed depends among other things on the activity or effective concentration of the water available. The equilibrium between water and protein is reversible. Proteins in equilibrium with water at high activity values will give up water, if the activity of the water in the system is brought to low values. The curve connecting the activity of the water with the amount held by the protein is not, however, a simple, smooth curve. The water appears to go in or out of the system in two stages and over a wide range is independent of the activity.

Katz (1917) studied the amount of water held by casein and other proteins in atmospheres of varying degrees of saturation. In atmospheres more than half saturated (that is, with activity values > 0.5), the amount held was a function of the activity; in atmospheres one-half to one-fifth saturated (activities 0.5 to 0.2), the amount held was practically constant, while in atmospheres less than one-fifth saturated, the amount again fell with decreasing saturation.

Water can be drawn out of moistened proteins by other means than evaporation. Freezing is an effective method and has been used by Moran (1926, 1932, 1935). Moran has measured the amount of water held by a number of proteins against the temperature at which ice crystals begin to form, that is, the temperature at which the forces binding water to water in the crystal lattice of ice, become greater than the forces binding water to protein. He also finds that the water held in protein systems can be considered under two headings—loosely bound water, the amount of which depends on the temperature but which is removed by freezing to $-20°$, and firmly bound water which resists freezing apparently to temperatures as low as $-100°$.

Water can also be expelled from protein systems by direct pressure, a method used by Jordan Lloyd and Moran (1934) for studying the relations of water in iso-electric gelatin gels. Here again the same relation is found; the water in the gel comes out in two stages. The amount of loosely bound water held in the gel is a function of pressure,

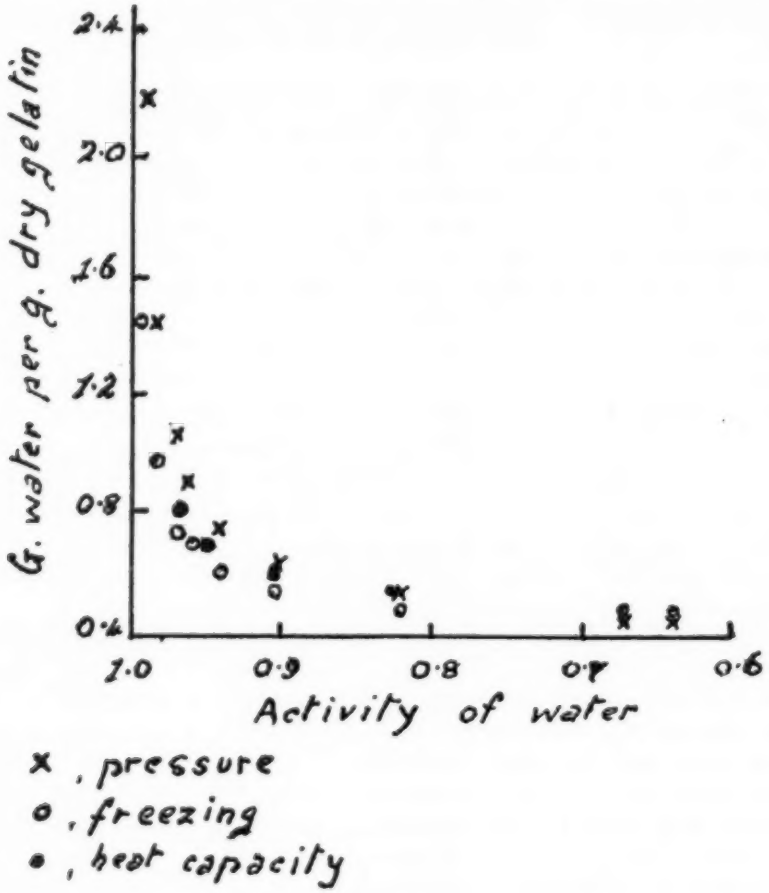


Figure 1.

falling with increasing pressure, but apparently completely removed by a pressure of 7,000 pounds per square inch. At pressures from 7,000 to about 30,000 pounds per square inch, the amount of water held is very nearly constant, but at higher pressures, still more water, namely, the firmly bound water, begins to leave the system.

The variation of the activity of water with vapor pressure, temperature and pressure can be calculated. If the amount of water

held per gram of dry protein is plotted against the activity of the water, all three methods give remarkably consistent results. The bound water can also be measured by calorimetric methods (Hampton and Mennie, 1932). The results obtained for iso-electric gelatin by pressure, freezing, and heat capacity measurements are shown in

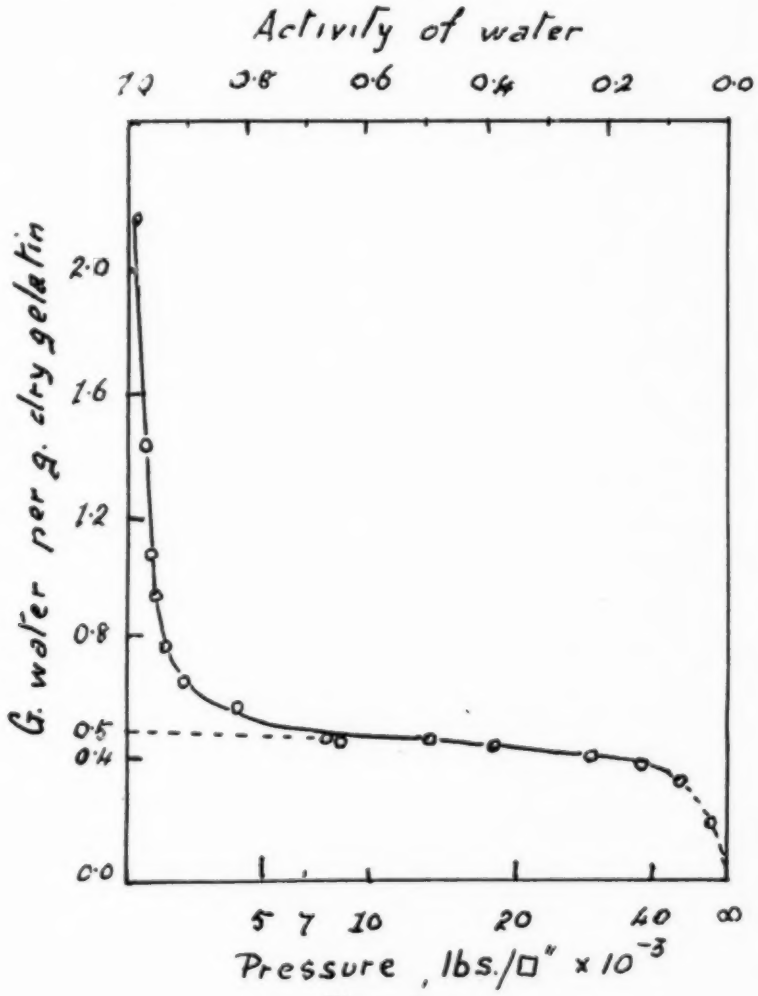


Figure 2.

Figure 1. This figure only shows bound water over the range of activities from 1.0 to 0.6. Figure 2 shows the bound water plotted against pressure for gelatin over the full range of activity. This shows very clearly that for a wide range of activities, the bound water of gelatin is constant at 0.5 gram per gram of dry gelatin.

The activity of water at which the loosely bound water is given up appears to be much the same for a number of proteins. This is shown in Figure 3, which illustrates results obtained by Moran (1935) in

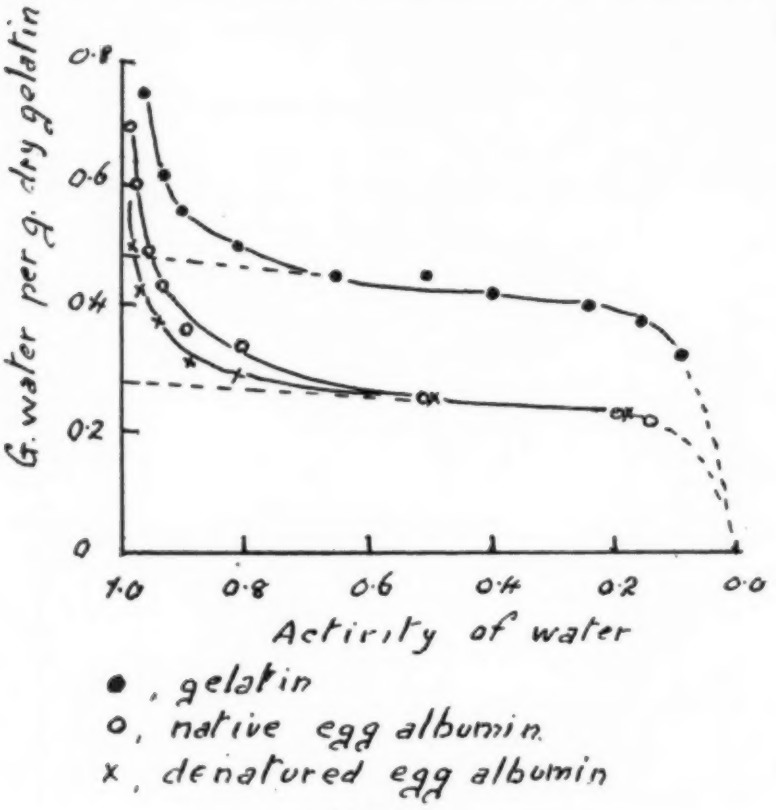


Figure 3.

freezing experiments with gelatin, native egg albumin and denatured egg albumin. The actual amount of water bound differs in the different proteins. Moran (1935) gives the following figures for firmly bound, or, as he now calls it, chemically bound water:

Gelatin, 0.5 g. water per 1 g. dry protein.
Native egg albumin, 0.26 g. water per 1 g. dry protein.
Denatured egg albumin, 0.26 g. water per 1 g. dry protein.
Myogen, 0.4 g. water per 1 g. dry protein (approximately).
Total solids of muscle, 0.33 g. (0.15 g., Brooks, 1934) water per 1 g. dry protein.

The amount of loosely bound water also differs in different proteins. Moran's experimental figures suggest that there is less loosely bound

water in denatured egg albumin than in native egg albumin, and that in myogen there is a comparatively small amount at all activities of water.

The evidence on the whole seems convincing that water is held to protein molecules by two different methods, one of which forms a much closer association than the other. There has been considerable discussion as how best to define "bound water." Probably the best definition is that it is that part of the water in a system which is no longer available for dissolving other solutes. This definition arises largely from the work of Gortner and his colleagues which has done so much toward showing both the theoretical and the practical importance of this aspect of protein science (Newton and Gortner, 1922, and Jones and Gortner, 1932). Gortner and his colleagues added a known amount of sucrose to expressed plant juices, and measured the resulting depression of the freezing point which was found to be greater than the value calculated on the assumption that all the water present was free to dissolve the sucrose. The obvious inference is that all of the water is not free, but that some is bound to the proteins present in the expressed juices. It should, perhaps, be mentioned here that Greenberg and Greenberg (1933) have added sucrose to protein solutions and then submitted them to ultra-filtration and state that the protein does not retain more than 10% of its own weight of water under these conditions. However, even if opinions differ as to the amount of bound water, and even if there is not as yet a generally accepted definition of bound water, there is no doubt that proteins can and do hold water in a very close association. A direct demonstration of this has been made by Hatschek (1936) who showed that a gelatin gel containing cobalt chloride showed the characteristic pink color of the hydrated cobalt salt, but that if it were allowed to dry slowly by evaporation, it turned to the blue color of the anhydrous cobalt salt, while there was still 33% of water in the gel.

Even though there is still much investigation needed, it may not be without profit to consider the possible forms which an association between water and protein might take.

In the first place, reference must be made to the work of Cohn and his colleagues at Harvard on the influence of proteins on the dielectric properties of water (see especially Cohn, 1936; Wyman, 1936). This, more than any other recent work, has led to the vivid realization of the protein molecule as a multipolar particle. On account of this multipolar structure, the molecule will be surrounded by complicated fields of force between the positive and negative poles (the so-called Coulomb forces), and the polar molecules of water will become orientated in the neighborhood of a protein molecule according to the strength and direction of these forces.

The strength of the Coulomb forces around a protein molecule will depend not only on the number of polar pairs, but also on their arrangement over the surface. Anything which leads to an alteration in their number or configuration will lead to an alteration in the number of polar molecules of water held around the protein molecule. Moran's (1935) recent work shows that denaturation leads to a reduction in the amount of loosely bound water, and Astbury has shown that denaturation is an alteration in the configuration of the molecule causing the open globular form of the water soluble protein to become more fibrous and compact (see especially Astbury and Lomax, 1935; Astbury, Dickinson, and Bailey, 1935). This might well lead to the positive and negative poles of the protein molecule being brought closer together with an accompanying reduction of the polar moment, and release of some of the bound water (see Cohn, McMeekin, Edsall, and Blanchard, 1933).

The amount of water held around a protein molecule by these interionic forces must obviously be affected by the nature, number, and distribution of any other ion pairs present. Moran has shown that the presence of salts reduces the amount of loosely bound water, a result which would be expected if the molecules are held in the polar fields around all the ions present in the solution.

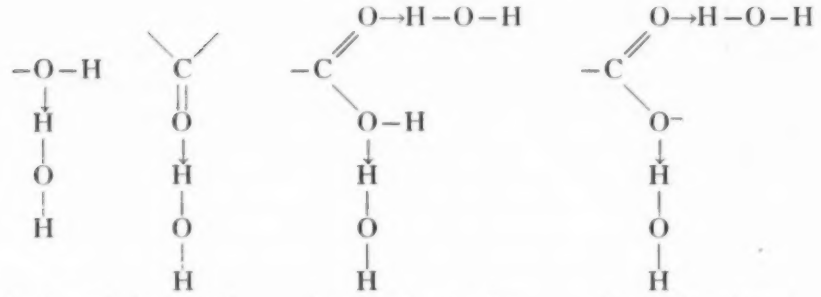
The effect of adding acids or alkalies to a protein system is interesting. It is well known that proteins are most easily precipitated by dehydrating agents, such as alcohol, at their iso-electric points, and Hardy, as long ago as 1905, suggested that electrically charged proteins were more heavily hydrated than those which are iso-electric to the surrounding medium. The addition of acid or alkali to a protein, by suppressing one member of a zwitterion pair, will undoubtedly alter the polar configuration of the protein molecule. Moreover, the prevailing charged centers of the protein molecule (positive in acid solutions and negative in alkaline ones) will now have as their gegenions comparatively small ions of possibly the same or possibly a different valency. This in itself will lead to alteration in the localization of the water around the protein molecule, and if Fajan's principle holds, would lead to an increase in the amount of loosely bound water, the increase being greater for monobasic than for dibasic acids, which appears to be the case (see Jordan Lloyd and Phillips, 1933).

The balance of evidence with regard to the multipolar character of the protein zwitterions suggests therefore that this multipolar character is sufficient to account for the loosely bound water of proteins—water which is not a fixed quantity per molecule of protein, but varies according to the general distribution of the interionic forces throughout the system.

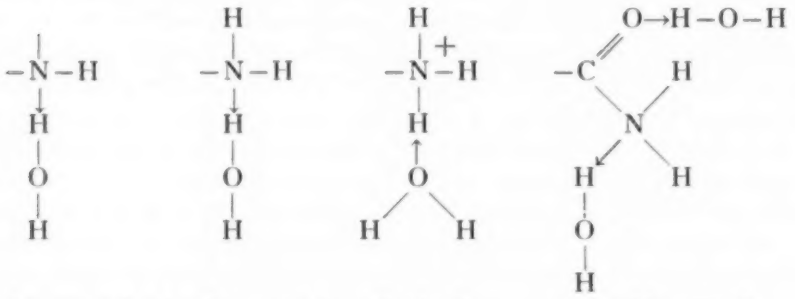
When we come to consider the firmly bound water, the matter assumes a somewhat different complexion. To begin with, although the evidence is scanty, what there is suggests that it is a much more constant quantity. Moran's (1926) experiments carried out by the freezing method suggest that the amount of the closely bound water is not altered by denaturing the protein. Moreover, other experiments suggest that it is not altered by the presence of salts (Moran, 1932). Some experiments carried out by my colleague, Dr. F. E. Humphries, give a figure of 0.46 g. of bound water per gram of dry gelatin at pH 2.6 compared with 0.48 g. in the iso-electric state. The water was removed by pressure and the figures indicate that the addition of acid to the gelatin has not altered the amount of closely bound water. The inference is, therefore, that neither altering the architectural pattern of the protein molecule nor altering the interionic forces throughout the system appreciably affects the closely bound water. A consideration of the constitution of the protein molecule shows that it must possess the capacity to coordinate with water molecules. For instance, where any of the side chains of the molecule contain oxygen or nitrogen atoms, there will be possibilities of coordination with water, and a protein containing a number of such side chains will have a greater affinity for water than a protein in which the side chains are mostly hydrocarbon chains. There are also oxygen and nitrogen atoms at every peptide link down the main polypeptide backbone, and the capacity to coordinate with water will be greater in protein systems where the molecules exist in the free state than in systems like fibers where they are held together in bundles by direct carbonyl-imino links between adjacent polypeptide chains.

The oxygen atoms of protein molecules occur in carbonyl, amide, or hydroxy groups, the nitrogen atoms in amide, imino, or amino groups. In all these groups the oxygen and nitrogen atoms possess electrons which are not utilized in covalent linkages and which may, therefore, coordinate with water molecules by the donation of electrons or formation of a hydrogen bond as indicated on top of page 32.

There is also the possibility of coordination with water molecules by the acceptance of electrons from the oxygen atoms of water molecules onto hydrogen atoms linked to nitrogen or oxygen atoms. This seems a less probable mode of coordination with the exception of the ionised amino group, where the presence of a positive charge will favor the acceptance of electrons. The number of water molecules which will be held in this way by each group can not be assessed, but is not likely to be large. Allowing one water molecule to each possible point of coordination on the side chains and backbones of the gelatin molecule, a rough calculation shows that taking the molecular weight



(hydroxyl group) (carbonyl group) (carboxyl group) (ionised carboxyl group)



(imino group) (amino group) (ionised amino group) (amide group)

of gelatin at 34,500, one molecule could coordinate with about 900 water molecules. One gram of dry gelatin holds 0.5 g. of closely bound water which gives a figure of 960 water molecules to one molecule of gelatin. These figures, though rough, encourage the belief that closely bound water is chemically bound to the protein. Coordination with water by the donation or acceptance of electrons is characteristic of uncharged groups, and will, therefore, occur at all values of hydrogen-ion concentration or salt concentration. Further evidence of the chemical nature of the binding may be deduced from the observation that the chemically bound water of egg albumin corresponds closely to the water of egg albumin crystals (Moran, 1935).

The problem of bound water is one of considerable importance, both in animal and plant physiology. It is not fixed throughout the life of the animal or plant. For instance, the amount of bound water is greatest in young animals and decreases with age. In plants it may also vary with the season.

It is hardly necessary to remind a meeting of cereal chemists of the work of Newton and Gortner (1922) on bound water and winter hardiness in wheats. The wheat Minhardy which has a high per-

centage of bound water in its winter leaves can resist the freezing temperatures of the cold season; the wheat Fulcaster, in which the leaf proteins have a lower percentage of bound water, can not do so. A high percentage of bound water enables a plant to resist desiccation not only by freezing, but also by drouth, as was shown by Newton and Martin (1930) in their work on drouth-resistant grasses.

Summary

Proteins imbibe water and hold it in two different forms which may be distinguished as loosely bound and firmly bound water.

The amount of total water bound varies with the activity of the water in the system.

The loosely bound water falls with decreasing activity of water, until the latter falls to about 0.7. At lower activities, 0.7 to 0.2, only the firmly bound water is present, which remains constant in amount over this range. Below an activity of 0.2, the firmly bound water is given up.

The loosely bound water can be removed by freezing, by direct pressure, or by evaporation.

The forces holding the loosely bound water are the interionic forces arising from the multipolar nature of the protein zwitterion.

It is suggested that the forces holding the closely bound water are chemical in nature, and are due to the formation of a coordinate link or hydrogen bond between the water molecules and the oxygen and nitrogen atoms of the protein. It is suggested that the presence of direct carbonyl-imino linkages between the polypeptide backbones of adjacent molecules, such as occur in fibers, reduces the amount of closely bound water.

The significance of these results in animal and plant physiology is indicated.

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REPORT OF THE 1936-37 COMMITTEE ON TESTING BISCUIT AND CRACKER FLOURS

WALTER REIMAN, *Chairman*

Kroger Food Foundation, Cincinnati, Ohio

(Read at the Annual Meeting, May 1937)

The report of the 1935–36 Committee on Testing Biscuit and Cracker Flours (Cereal Chem. **13**: 755–769) recommended that:

> Work be repeated on other flours, subjecting the viscosity and baking tests to further examination employing essentially the technique therein outlined, and that a start be made to establish a definite correlation between laboratory and actual shop results by means of carefully controlled laboratory tests and practical tests on a commercial basis.

The present committee directed its activities toward carrying out the recommendations of the previous committee.

Plan of Procedure

Four flours representing respectively the maximum and minimum ranges for cracker dough and cracker sponge flours, as measured by the MacMichael viscosimeter (see Bohn, 1935), were selected for laboratory testing by members of the committee and also for the commercial shop tests.

The four flours were twice submitted to the collaborators for testing, but were coded so as not to reveal that identical flours were being tested in each series.

On the first series of tests the committee members were asked to determine the moisture, ash and protein contents of the flours, to measure their viscosity, and to classify the flours as to cracker dough, cracker sponge, or biscuit types on the basis of these results.

For the second series of tests the collaborators were asked to bake duplicate "pup" loaves from the flours and again to classify the flours on the basis of the baking test.

In order to check the classification of the flours on the basis of the baking test, loaves were baked from the four flours at the Kroger Food Foundation and sent to three members of the committee who were asked to score the loaves and classify the flours from which they were baked using the same methods of interpretation they applied to their own loaves.

For the cracker bakes on the commercial shop scale, each of the two sponge flours was combined with each of the two dough flours to

make four different types of cracker mixes. These cracker bakes were made three times each, with intervals of one month between the three series of bakes. A one-pound package of each type of cracker was sent to the collaborators for scoring after each bake. A chemical and physical analysis was also made on the four crackers of each bake at the Kroger Food Foundation.

Materials and Methods of Study

Materials Used

The four flours used in this investigation and which shall be designated as Nos. 1, 2, 3, and 4 were of the following types.

No. 1. A strong soft winter wheat flour milled from southern Illinois wheat. It is generally used as a cracker sponge flour and represents the maximum in the viscosity range for this type of work.

No. 2. A moderately strong soft winter wheat flour milled from northern Indiana wheat. Normally finds its best application as a cracker sponge flour but is also satisfactory as a cracker dough or cookie flour. This flour is representative of the minimum in the viscosity range for cracker sponge flours.

No. 3. This is a weak soft winter wheat flour milled from central Ohio wheat. This flour, which is representative of the softest type of cracker dough flour, gives its best performance when used as such or as a cookie flour.

No. 4. This is a moderately strong soft winter wheat flour but not as strong as No. 2. This flour which was milled from southern Indiana wheat is representative of the strongest type of cracker dough flour and performs best when used for this purpose or as a cookie flour.

Methods of Study

Baking test: The baking test used was that of Werner, as described in Cereal Chemistry, volume 5, page 158, with variations as noted under Method B, Cereal Chemistry, volume 13, page 757.

The terminology used in describing the test loaves is also given under the last reference cited above.

Viscosity test: The viscosity test used was procedure No. 2, as suggested by Bayfield in Cereal Chemistry, volume 13, page 786.

Shortometer test: The Bailey Shortometer was used in this work. The shortometer readings recorded in the tables are in units of 1/32 pounds.

Commercial Scale Cracker Bakes

All of the crackers were baked on the basis of 5 barrels of flour to each mix. In each unit, 50% of the flour was of the cracker sponge type and 50% was of the cracker dough type.

Sixty percent or 3 barrels of the total amount of flour used went into the sponge along with 54% of the shortener, about 85% of the water, and all of the yeast. The 3 barrels of flour in the sponge were composed of 2½ barrels, or all of the sponge-type flour and 1/2 barrel or 20% of the dough-type flour. On the first two bakes, which are

designated respectively as A and B in this report, the sponges were fermented for 20 hours at 78° F. and 60 to 66% relative humidity. Due to the shop schedule at the time of the third bake, designated as bake C in this report, the sponge time was reduced to 17 hours. The temperature and relative humidity were the same as on bakes A and B.

To make up the dough, the fermented sponge was mixed with the two remaining barrels of the dough-type flour, 46% of the shortener, salt, malt, enough water to produce the proper dough consistency, and sodium bicarbonate. The quantity of sodium bicarbonate used was varied within narrow limits according to the temperature of the fermented sponge. The doughs were proofed for four hours and then baked into 2-inch square crackers at 540° F.

The crackers from each bake were scored by the collaborators, using the following method of evaluation.

CRACKER SCORING

	Appearance 20 points
3 Symmetry of edge	3 Even, 2 uneven, 1 fish mouth, burnt
3 Shape	3 Even, 2 uneven, 1 buckled-cupped
5 Eveness of bake and color	5 Even, 3 uneven, 1 very uneven
3 Top color	3 Excellent, 2 fair, 1 poor
3 Bottom color	3 Good, 2 fair, 1 burnt, 0 dirty, floury
3 Bottom appearance	3 Good blisters, 2 fair, 1 too few, too many
	Texture 50 points
10 Flakiness	10 Very flaky, 8 flaky, 6 compact, 3 very compact
15 Crispness	15 Crisp, 10 soft, 5 very soft
15 Tenderness	15 Very tender, 12 tender, 8 tough or flinty, 4 very tough
5 Chipping	5 Not chippy, 3 slightly chippy, 1 chippy
5 Color of crumb	5 White, 3 creamy, 1 grey
	Flavor 30 points
20 Quality	20 Excellent, 15 good, 10 fair, 5 poor
5 Salt	5 Good, 3 too much, too little
5 Soda	5 Good, 3 too much, too little

In addition to scoring the crackers, the collaborators measured their pH by spotting them with either phenol red or brom thymol blue, determined the number of crackers per pound, and also the height of ten crackers.

An analysis for moisture, protein, and fat was made on each batch of crackers in addition to the shortometer test for tenderness. The shortometer figure recorded for each cracker represents the arithmetical average of 200 readings.

Experimental Results

Tables I, II, III, and IV record the analytical and viscosity results obtained by the collaborators on the four flours used in this investiga-

TABLE I

RESULTS OF COLLABORATIVE TESTS WITH FLOUR NO. 1

Collaborator	% H_2O	% Ash as received	% Ash at 15% H_2O	% Protein as received	% Protein at 15% H_2O	Viscosity ° McM.		
						No acid	1 c.c.	7 c.c.
Pearl Brown	11.18	0.373	0.357	9.72	9.30			
C. C. Armuth	10.50	0.376	0.357	9.79	9.30	18	69	92
C. F. Evert	11.50	0.36	0.346	9.42	9.05	10	19[1]	97
Jan Micka	9.90[1]	0.38	0.359	9.90	9.34	12	54	96
C. O. Oppen		0.365		9.78		10	50	94
Kroger Foundation	12.46	0.380	0.369	9.55	9.28	14	58	89
Mean	11.41	0.372	0.358	9.70	9.25	13	56	94
Maximum	12.46	0.380	0.369	9.90	9.34	18	69	97
Minimum	9.90	0.360	0.346	9.42	9.05	10	19	89

[1] Not included in averages.

Comments

Pearl Brown: "Too strong even for cracker sponges." [2]

Messrs. Armuth and Knudson: "A cracker sponge flour."

C. F. Evert: "A cracker sponge flour."

Jan Micka: "A medium strong cracker sponge flour. The shorter extraction is responsible for the very high viscosity."

C. O. Oppen: "A cracker sponge flour."

Kroger Foundation: "A satisfactory cracker sponge flour."

[2] From average of viscosities.

TABLE II

RESULTS OF COLLABORATIVE TESTS WITH FLOUR NO. 2

Collaborator	% H_2O	% Ash as received	% Ash at 15% H_2O	% Protein as received	% Protein at 15% H_2O	Viscosity ° McM.		
						No acid	1 c.c.	7 c.c.
Pearl Brown	11.09	0.345	0.330	8.36	8.00			
C. C. Armuth	10.60	0.346	0.329	8.37	7.96	18	48	62
C. F. Evert	11.10	0.334	0.320	8.15	7.79	10	45	60
Jan Micka	9.50[1]	0.350	0.329	8.54	8.02	16	40	60
C. O. Oppen		0.490[1]		8.40		11	38	63
Kroger Foundation	12.60	0.340	0.331	8.13	7.91	14	44	60
Mean	11.35	0.343	0.328	8.33	7.94	14	44	61
Maximum	12.60	0.490	0.320	8.54	8.02	18	48	63
Minimum	9.50	0.334	0.331	8.13	7.79	10	38	60

[1] Not included in averages

Comments

Pearl Brown: "A cracker sponge flour." [2]

Messrs. Armuth and Knudson: "Either a cracker sponge or cracker dough flour. Would probably work well in either case or as a topping flour for cracker dough sponge."

C. F. Evert: "A cracker sponge flour."

Jan Micka: "A cracker dough and cookie flour."

C. O. Oppen: "A cracker sponge or dough flour."

Kroger Foundation: "Best suited for use in cracker sponges but could also be used in cracker doughs."

[2] From average of viscosities.

TABLE III

RESULTS OF COLLABORATIVE TESTS WITH FLOUR NO. 3

Collaborator	% H_2O	% Ash as received	% Ash at 15% H_2O	% Protein as received	% Protein at 15% H_2O	Viscosity ° McM.		
						No acid	1 c.c.	7 c.c.
Pearl Brown	11.00	0.387	0.370	8.40	8.02			
C. C. Armuth	10.60	0.380	0.361	8.30	7.89	14	24	35
C. F. Evert	11.10	0.374	0.358	8.24	7.88	10	20	38
Jan Micka	9.80[1]	0.396	0.373	8.50	8.01	12	20	40
C. O. Oppen		0.410		8.42		8	16	35
Kroger Foundation	12.05	0.390	0.377	8.22	7.95	11	19	35
Mean	11.19	0.390	0.368	8.35	7.95	11	20	37
Maximum	12.05	0.410	0.377	8.50	8.02	14	24	40
Minimum	9.80	0.374	0.358	8.22	7.88	8	16	35

[1] Not included in averages.

Comments

Pearl Brown: "A cookie flour. This might border upon dough flour performance." [2]

Messrs. Armuth and Knudson: "A cracker dough flour."

C. F. Evert: "A cracker dough or cookie flour."

Jan Micka: "A cracker dough and cookie flour."

C. O. Oppen: "A cracker dough and cookie flour."

Kroger Foundation: "Can be used as a cracker dough flour and also for hard sweet goods."

[2] From average of viscosities.

TABLE IV

RESULTS OF COLLABORATIVE TESTS WITH FLOUR NO. 4

Collaborator	% H_2O	% Ash as received	% Ash at 15% H_2O	% Protein as received	% Protein at 15% H_2O	Viscosity ° McM.		
						No acid	1 c.c.	7 c.c.
Pearl Brown	11.39	0.397	0.381	8.44	8.10			
C. C. Armuth	10.60	0.384	0.365	8.42	8.01	14	35	60
C. F. Evert	11.70	0.373	0.359	8.20	7.89	10	24	58
Jan Micka	9.70[1]	0.402	0.378	8.64	8.14	10	25	60
C. O. Oppen		0.410		8.38		8	20	60
Kroger Foundation	12.78	0.380	0.370	8.16	7.96	11	28	55
Mean	11.62	0.391	0.371	8.37	8.02	11	26	59
Maximum	12.78	0.410	0.381	8.44	8.14	14	35	60
Minimum	9.70	0.373	0.359	8.16	7.89	8	20	55

[1] Not included in averages.

Comments

Pearl Brown: "A cracker dough flour. This might be a borderline flour in the direction of a cracker sponge type." [2]

Messrs. Armuth and Knudson: "A borderline cracker sponge flour. Would probably work better as a cracker dough flour or as a topping flour for cracker dough sponge."

C. F. Evert: "A cracker dough flour."

Jan Micka: "A stronger type cracker dough flour."

C. O. Oppen: "A cracker dough flour."

Kroger Foundation: "This flour is on the border between a cracker sponge and cracker dough type, but would probably be more satisfactory when used for cracker doughs."

[2] From average of viscosities.

tion. The classifications assigned to the flours, on the basis of the viscosity test, are also given in these tables. Tables V and VI report

TABLE V

BREAD SCORES ON FLOURS 1 AND 2

Characteristic	Flour 1				Flour 2			
	Pearl Brown	C. F. Evert	C. O. Oppen	Kroger Foundation	Pearl Brown	C. F. Evert	C. O. Oppen	Kroger Foundation
Oven spring	Medium large	Small	Medium	Large	Large	Small	Medium	Large
Break and shred	Ragged	Slightly ragged	Fairly smooth	Slightly shell top	Very ragged	Slightly ragged	Torn	Fairly smooth
Crust color	Dark	Pale	Pale	Medium dark	Pale	Pale	Pale	Medium dark
Grain	Close, elongated	Close and elongated	Close, elongated	Close, elongated	Close, round	Coarse and elongated	Close, round	Close, elongated
Color of crumb	White gray	White	White	White	Cream gray	Slightly dull	Creamy white	White
Volume c.c.		460	485	415		445	480	415
% Absorption	57	56	56	57	55	54	55	57

Comments

Pearl Brown—"A strong sponge type flour."

C. F. Evert—"Suitable for cracker sponging."

C. O. Oppen—"This is definitely a cracker sponge flour."

Kroger Foundation—"The strongest of the four flours. Satisfactory for use in cracker sponges."

"A cracker dough flour but strong enough for use in cracker sponges."

"Loaves baked from flours 2, 3, and 4 are so similar we can make no distinction between the flours."

"Satisfactory for use in both cracker sponges and cracker doughs."

"Not as strong as 1 but strong enough for use as a cracker sponge flour."

TABLE VI

BREAD SCORES ON FLOURS 3 AND 4

Characteristic	Flour 3				Flour 4			
	Pearl Brown	C. F. Evert	C. O. Oppen	Kroger Foundation	Pearl Brown	C. F. Evert	C. O. Oppen	Kroger Foundation
Oven spring	Medium large	Small	Medium	Large	Large	Small	Small	Large
Break and shred	Very ragged	Slightly ragged	Torn	Torn	Ragged	Slightly ragged	Slight	Fairly smooth
Crust color	Medium	Pale	Pale	Medium	Dark	Pale	Pale	Medium
Grain	Coarse	Slightly coarse	Close, round	Close, elongated	Very coarse	Coarse	Coarse	Close, elongated
Color of crumb	Cream	Dull	White	Creamy white	Dark cream	Dull	Dull creamy white	Creamy
Volume, c.c.		450	480	440		452	450	405
% Absorption	55	55	55	57	55	54	56	57

Comments

Pearl Brown—"A cookie flour."

C. F. Evert—See Table V.

C. O. Oppen—"Could be successfully used in both cracker sponges and doughs."

Kroger Foundation—"Stronger than 4, but softer than either 1 or 2. Most applicable for use as a cracker doughing flour."

"A cookie flour."

See Table V.

"A cracker doughing flour."

"The softest of the four flours. Suitable for use in cracker doughs and hard sweet goods."

the collaborators' scores on their own test loaves, while Tables VII and VIII record their scores on the loaves baked from the same flours

TABLE VII

COLLABORATORS' SCORES ON SAME LOAVES

Characteristic	Flour 1			Flour 2		
	Pearl Brown	C. F. Evert	C. O. Oppen	Pearl Brown	C. F. Evert	C. O. Oppen
Oven spring	Large	Small	Medium	Medium large	Small	Medium
Break and shred	Ragged	Ragged	Slightly torn	Ragged	Slightly ragged	Torn
Crust color	Medium	Medium	Medium	Slightly dark	Medium	Medium
Grain	Elongated	Close and elongated	Elongated	Close and elongated	Close and elongated	Small round
Color of crumb	White	White	Creamy white	Creamy white	Slightly dull	White
Volume c.c.		360	370		370	395
% Absorption		57			57	

Comments

Pearl Brown—"A cracker dough flour. Could also be used in the sponge." "A cracker sponge flour."

C. O. Oppen—"A cracker sponge flour." "A cracker dough flour."

TABLE VIII

COLLABORATORS' SCORES ON SAME LOAVES

Characteristic	Flour 3			Flour 4		
	Pearl Brown	C. F. Evert	C. O. Oppen	Pearl Brown	C. F. Evert	C. O. Oppen
Oven spring	Large	Small	Medium	Medium, large	Small	Medium
Break and shred	Very ragged	Shell	Torn	Very ragged	Smooth	Torn
Crust color	Pale	Pale	Pale	Dark	Medium	Brown
Grain	Coarse	Slightly coarse	Coarse	Elongated	Close and round	Small round
Color of crumb	Creamy gray	Dull	Dull white	Gray white	Dull	Creamy dull
Volume c.c.		355	380		370	375
% Absorption		57			57	

Comments

Pearl Brown—"A cookie flour." "A cookie flour."

C. O. Oppen—"A cracker dough or sweet goods flour." "A cookie flour."

at the Kroger Food Foundation. These tables also include the collaborators' classifications of the flours on the basis of the baking test. Figures 1 and 2 are the photographs of the test loaves baked by the collaborators from the four flours used in this work.

The chemical and physical measurements of the crackers from each of the three bakes and of the flours as used at each bake are reported in Table IX. Table X summarizes the ranking of the crackers of each bake by means of the collaborators' scores and the shortometer readings.

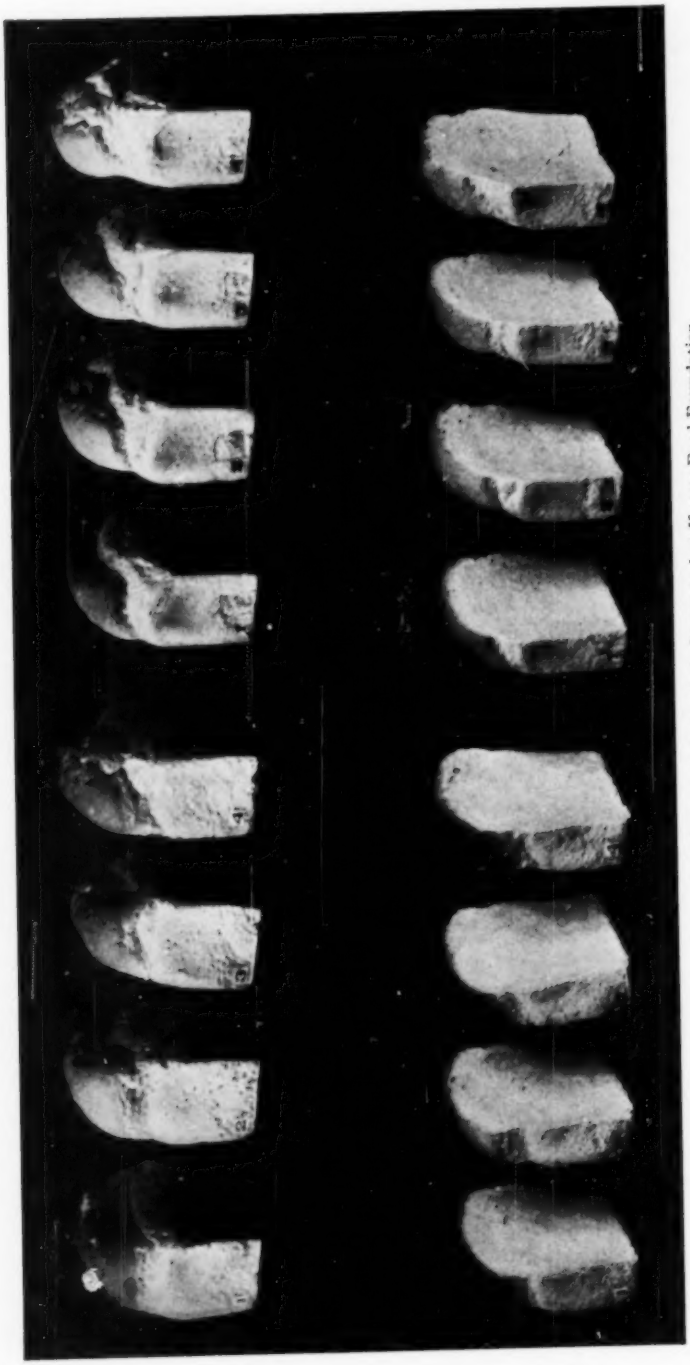

Figure 1. Loaves baked respectively by C. O. Oppen and at Kroger Food Foundation.

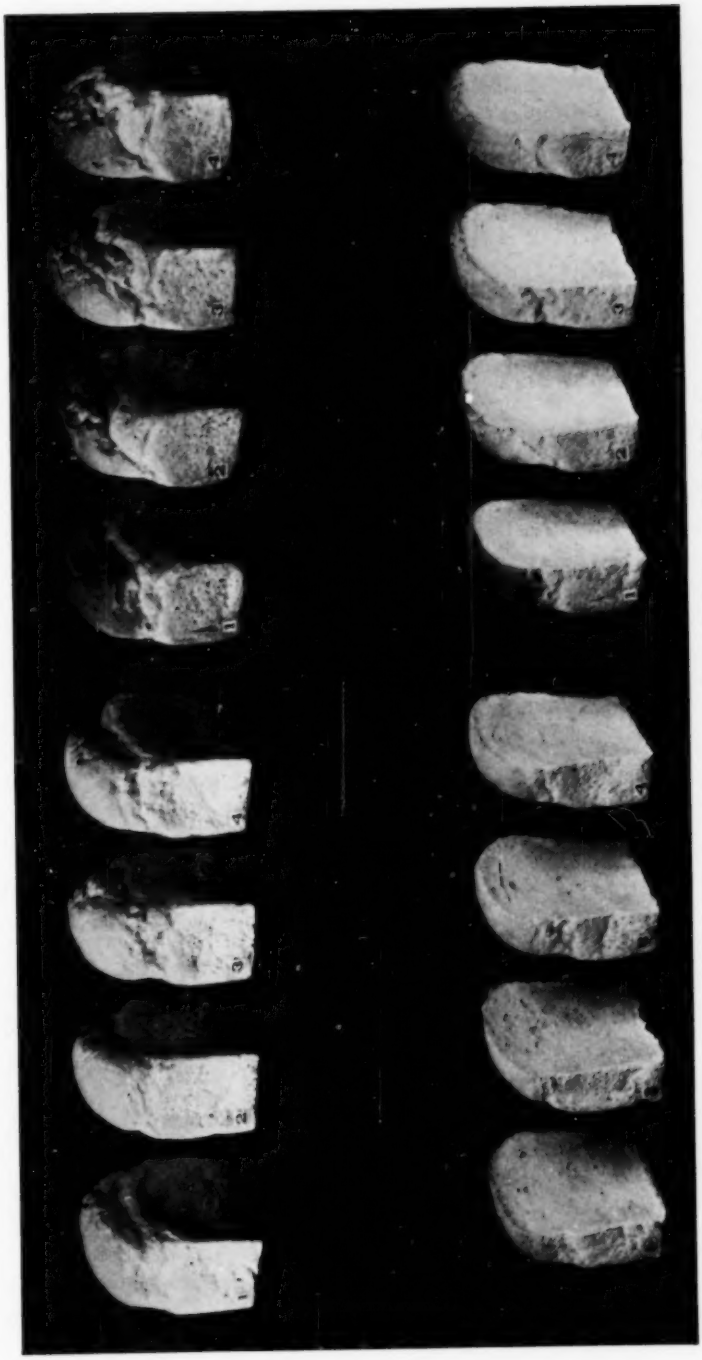

Figure 2. Loaves baked respectively by C. F. Evert and Pearl Brown.

TABLE IX

CHEMICAL AND PHYSICAL MEASUREMENTS ON CRACKERS AND ON FLOUR AT EACH BAKE

Crackers									Flour					
Bake	Cracker	% Moisture	% Protein	% Fat	Shortometer reading[1]	pH	Thickness 10 (in.)	No./lb.	No.	% Moisture	% Ash	% Protein	Viscosity: No acid	Viscosity: 1 c.c.	Viscosity: 7 c.c.
A	1	4.16	8.54	13.48	79.5	7.4	2 5/8	108	1	11.68	0.40	9.89	14	62	96
	2	4.60	8.23	13.22	88.8	8.2	3 1/8	110	2	11.84	0.40	8.11	14	46	60
	3	3.36	7.88	13.55	76.6	7.6	2 3/4	114	3	11.74	0.39	8.27	11	24	36
	4	3.38	7.95	13.37	84.5	7.8	2 5/8	114	4	12.61	0.35	7.80	12	30	54
B	1	4.41	8.42	12.83	89.4	7.4	2 5/8	108	1	12.55	0.38	9.40	14	62	95
	2	5.63	8.23	12.66	96.6	7.4	3	109	2	12.73	0.34	7.94	15	44	55
	3	3.70	7.75	13.04	74.8	7.4	2 3/4	112	3	12.53	0.39	8.10	9	23	36
	4	4.02	7.60	13.12	77.7	7.4	2 3/4	113	4	12.94	0.39	8.12	11	24	53
C	1	5.12	8.50	12.54	91.4	8.4	3	108	1	13.16	0.38	9.47	14	59	98
	2	4.31	8.42	12.85	85.4	7.6	3 1/8	111	2	13.75	0.35	7.78	15	44	57
	3	4.34	7.80	13.06	81.1	8.2	2 7/8	111	3	12.82	0.41	8.07	11	21	35
	4	4.28	7.68	12.84	82.9	8.2	3 1/8	109	4	13.65	0.41	7.98	10	33	57

Fat on flour				
No.	1	2	3	4
% Fat 15% H_2O	0.48	0.91	1.10	0.80

[1] Unit of measurement 1/32 pound.

TABLE X

RANKING OF CRACKERS FROM COLLABORATORS' SCORES AND SHORTOMETER TESTS

	Bake A				*Bake B*				*Bake C*			
Ranking on Cracker No.	1	2	3	4	1	2	3	4	1	2	3	4
Pearl Brown	1	4	2	3	2	3	1	3	3	2	1	4
C. C. Armuth	2	4	2	1	2	1	2	2	2	1	2	4
C. F. Evert	2	4	3	1	4	1	2	3	3	2	4	1
Jan Micka					2	4	1	3	3	4	1	1
C. O. Oppen					3	4	1	3				
W. S. Culver	1	1	2	1	3	4	1	2	4	2	1	2
W. Reiman	3	4	1	2	2	4	1	3	3	4	1	2
Total	9	17	10	8	18	21	9	19	22	15	10	14
Rank	2	4	3	1	2	4	1	3	4	3	1	2

Shortometer

	Bake A				*Bake B*				*Bake C*			
Ranking on Cracker	1	2	3	4	1	2	3	4	1	2	3	4
Rank	2	4	1	3	3	4	1	2	4	3	1	2

Composite of Score and Shortometer Readings

Test	Collaborator's ranking				Shortometer ranking			
Cracker No.	1	2	3	4	1	2	3	4
Total	8	11	5	6	9	11	3	7
Rank	3	4	1	2	3	4	1	2

Discussion of Results

The analytical results reported by the collaborators on the flours used in this investigation are in good agreement with the exception of the figures on moisture content. The discrepancies in the moisture results on the flours do not reflect weaknesses in the analytical procedure but rather the condition encountered in transit and in the laboratories while awaiting analysis.

The ash results indicate that flour No. 2 is lowest on ash, flours Nos. 3 and 4 are about the same and highest on ash, with flour No. 1 in between these two extremes but closer to the higher figure. The figures on protein content show flour No. 1 to be about 1.3% higher on protein than flours Nos. 2, 3, and 4, which are practically the same.

With the exception of flour No. 1, the viscosity results reported for the flours are in fairly good agreement, with the magnitude of the difference between collaborators' readings on the same flour about like that reported in this work last year (*loc. cit.*). The only reason that the viscosity figures on flour No. 1 show a greater difference than on the other flours is because of the low reading reported by one collaborator. That this reading is an exception is indicated by the viscosity results on flour No. 1 reported by this same collaborator in Table IX. These figures compare favorably with the other results and if used would reduce the magnitude of the difference in viscosity readings to within that reported on flours Nos. 2, 3, and 4. In using their viscosity results as a means of classifying the flours, the collaborators were able to arrive at almost unanimous opinions on the best use for each flour.

The results of scoring the test loaves, reported in Tables V and VI, show satisfactory agreement (except for the figures on volume) among the various collaborators. The differences in the figures on volume may be explained in part by the fact that all the collaborators did not use the same size of baking pan. Individual differences in molding and panning technique may also contribute to this condition. Despite the differences in appearance of the loaves as shown in Figures 1 and 2, the collaborators were able to reach practically the same conclusions regarding the best commercial application for each flour. However, two of the collaborators who participated in both the viscosity and baking tests reported that flour No. 4 was stronger than flour No. 3, on the basis of the viscosity test, but on the basis of their baking results, said that flour No. 3 was stronger than No. 4. This condition is analogous to that reported by this committee last year (*loc. cit.*) where two of the flours were ranked a degree higher in strength by means of the baking test than by the viscosity test.

Tables VII and VIII indicate that the collaborators were able to reach the same conclusions with respect to the flours from loaves baked by someone else as from their own loaves.

Considering the number of variables in commercial cracker baking, the analyses of the four crackers at the times of their respective bakes, as reported in Table IX, show a fair degree of consistency. This is exhibited by the trends in the results rather than in the absolute values themselves. Crackers Nos. 1 and 2 have, in the instance of each bake, a higher percentage of moisture and protein and a lower percentage of fat than crackers Nos. 3 and 4. The shortometer readings show higher results on each bake for crackers Nos. 1 and 2 than for crackers Nos. 3 and 4. The results also show that crackers Nos. 1 and 2 have greater spring as evidenced by their thickness and run fewer to the pound than crackers Nos. 3 and 4. On the second bake only were the pH readings of the four crackers the same. The analysis of the flours at the time of the different bakes indicates that they gained moisture while in storage but remained practically constant on viscosity. The percentages of ash and protein decrease with the increase in moisture.

Although the crackers from each bake were scored in detail by the collaborators, these results are not presented since they would only tend to confuse rather than clarify the work. The reason for this is the rather marked differences of opinion expressed by the collaborators in their numerical evaluations of cracker characteristics. Table X however, records the relative rankings of the crackers based on the collaborators' scores in comparison with the relative rankings on the basis of the shortometer test. The cracker which scored highest and gave the lowest shortometer reading was ranked first on the respective bases. When compared with one another, on the basis of collaborators' scores, the rankings of the crackers at the time of each bake do not show a very great degree of consistency. More comparable rankings are reported on the basis of the shortometer tests. On only one bake, the third, do the cracker rankings, on the basis of collaborators' scores and shortometer tests, exactly agree. However, when the rankings at the time of each bake are composited on their respective bases the ranking of the crackers by means of the collaborators' scores and the shortometer tests is exactly the same. These show that cracker No. 3 is first, No. 4 second, No. 1 third, and No. 2 fourth.

On the basis of the viscosity test, cracker No. 3, which ranked first, is composed of the softer sponge and the softer dough flour; cracker No. 4, which ranked second, is made from the softer sponge and the stronger dough flour; cracker No. 1, which ranked third, is com-

posed of the stronger sponge and the softer dough flour; and cracker No. 2, which ranked fourth, is composed of the stronger sponge and the stronger dough flour.

Using the baking test as the criterion of strength, cracker No. 3 is composed of the softer sponge and stronger dough flour; cracker No. 4 is made from the softer sponge and the softer dough flour; cracker No. 1 is composed of both the stronger sponge and dough flours; and cracker No. 2 is made from the stronger sponge and softer dough flours. On the basis of either the baking or viscosity tests, as the measurement of flour strength, the quality of the crackers seems to be more dependent on the type of sponge flour than on the type of dough flour used in its formula.

A comparison of Tables IX and X shows that the best crackers have the least spring and lowest moisture content. This relationship is dependent on the types of flour used, since the best crackers were made from the softer flours which could be more easily baked out. The pH of a cracker is a determining factor in its spring, but the comparison still holds in bake B where all four crackers had the same pH.

Conclusions

As a result of its work this year, the Committee on Testing Biscuit and Cracker Flours concludes that:

While the relationship between cracker quality and types of flour as pointed out in this report is not definite nor are the data presented extensive enough to form precise conclusions, we believe there is sufficient evidence to indicate that the methods used in evaluating cracker flours give useful information and should be continued.

Further confirmation is given to the work of previous committees in their efforts to determine the relative strengths of biscuit and cracker flours by means of the baking and/or viscosity tests.

Recommendations

The committee therefore recommends that:

More work of the type herein reported be done to build up a greater volume of data to correlate more accurately the laboratory analyses of biscuit and cracker flours with their shop performance.

Experimental work be undertaken to explain and, if possible, correct the apparent discrepancy between the baking and viscosity tests for determining the relative strengths of biscuit and cracker flours.

Acknowledgments

This report is the result of the cooperative efforts of a number of individuals and appreciation for their work is hereby expressed. The

members of the committee who participated in the collaborative tests were: Miss Pearl Brown, C. C. Armuth, C. F. Evert, Jan Micka, C. O. Oppen, and the writer. Thanks are also due R. M. Knudson of Ogden, Utah for his work on the viscosity test.

The writer is indebted to George Garnatz for his suggestions in planning this work and much help in writing the report and to W. S. Culver for conducting the shop tests and making many of the analyses. Appreciation for preparing this report is due Mrs. Dorothy Shriver. The work of the Cereal Laboratory of the Kroger Food Foundation on the baking tests is acknowledged.

The flours used in this investigation were obtained through the efforts of H. H. Wurtz, Purchasing Agent for the Kroger Grocery and Baking Company. The shop tests were conducted at Kroger's Columbus Cracker Bakery through the courtesy of R. F. Lovell.

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THE QUANTITATIVE DETERMINATION OF BROMINE IN BROMATED FLOURS[1]

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(Read at the Annual Meeting, May 1937)

Recently this laboratory had occasion to investigate an extended series of competing flours obtained from several countries where the baking methods in use call for small percentages of yeast. When baked by the A. A. C. C. basic procedure many of the samples yielded loaves possessing decided overfermented characteristics, indicating that the flours had been heavily treated to increase the rate of gluten development and thereby render them more suitable for the baking procedures for which they were intended. Qualitative tests showed that these samples had been bromated and it became of importance to determine the quantities present.

As far as the quantitative determination of bromine in flour or flour improvers is concerned, only two methods, that of Meyer (1931) and of Kulman (1934, 1934a) were found in the literature. The method of Meyer (1931) which is described in the A. A. C. C. Book of Methods (1935) is a macro-volumetric method intended for the determination of bromates in commercial flour improvers; on the other hand, Kulman's method is described for treated flours containing 0.005% $KBrO_3$ and depends upon its separation, along with other added chemicals such as persulphates and iodates, by a flotation procedure involving centrifuging a suspension of the sample in chloroform or carbon tetrachloride. The quantity of potassium bromate isolated is determined by treatment with KI and HCl, overtitration of the liberated iodine with 0.01N $Na_2S_2O_3$ and the excess back-titrated with 0.01N KI. The presence of $KBrO_3$ must be identified before the method can be applied, and in flours containing persulphates or iodates these constituents must be determined separately in aliquots of the final solution.

During the last few years considerable effort has been devoted to the development of reliable micro methods for the estimation of bromine in biological material but it is not proposed to give here a complete survey of the extensive literature.

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[1] Published as paper No. 123 of the Associate Committee on Grain Research, National Research Council of Canada and Dominion Department of Agriculture.

All the procedures involve an initial destruction of the organic matter by wet or dry ashing followed by selective oxidation of the inorganic halides, the quantitative separation of bromine and its determination by titrimetric or colorimetric methods. A study of the literature reveals that the problem of bromine estimation in such materials is by no means simple and recent research has shown that a number of factors including loss of bromide during ashing, its incomplete oxidation, partial oxidation of the chlorides present and incomplete separation of the bromine, rendered many of the earlier methods unreliable. While the separation of bromides and iodides may be readily accomplished with a variety of oxidizing agents, the former being converted to bromine and the latter to iodate, the separation from chlorides is much more difficult and it is only recently that selective oxidation of chlorides and bromides has been satisfactorily accomplished.

Bobtelsky and Rosowskaya-Rossienskaya (1931) and Evans (1931) found that bromide may be rapidly and completely oxidized to bromine without oxidation of chlorides in a chromic-sulphuric acid mixture of definite concentration, and Yates' (1933) micro method for the determination of bromine in blood is based on this principle. Protein is first removed by precipitation with tungstic acid, the filtrate treated with potassium hydroxide, evaporated to dryness and incinerated at 500° C. for 20 minutes in a nickel crucible. The mixed halides are then oxidized with a chromic-sulphuric acid mixture of such concentration that the bromide is completely oxidized to bromine in the cold, while the chloride remains unaffected and any iodide present is converted to iodate. The bromine is removed by aeration, absorbed in starch-iodide solution and the liberated iodine titrated with 0.001N $Na_2S_2O_3$ from a microburette. Yates states that the method is accurate to within 2 γ [2] for amounts ranging from 5 to 1,000 γ of bromine.

For the micro determination of bromine in blood, Francis and Harvey (1933) destroy the organic matter by ignition with KOH under controlled conditions and employ a chromic-phosphoric acid mixture for liberation of bromine, which is recovered by aeration in all-glass apparatus and determined in the usual manner by absorption in starch iodide solution and titration of the liberated iodine with 0.002N $Na_2S_2O_3$. These workers report a recovery of bromine within $\pm$ 10% of the theoretical and emphasize the necessity of pure reagents, scrupulous cleanliness and attention to details. High results may be obtained with materials containing iodine.

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[2] One gamma equals one one-thousandth of a milligram.

Leipert and Watzlawek (1934) destroy organic matter by a wet ashing procedure using a chromic-sulphuric acid mixture in a closed apparatus, the liberated bromine and chlorine being led into NaOH solution where the bromide and hypobromite are oxidized by hypochlorite to bromate which is determined iodometrically.

Several colorimetric methods are also described in the literature. Scott (1925) states that small amounts of bromine may be determined colorimetrically by means of magenta reagent which produces a violet to reddish-violet coloration in the presence of bromine. Tod (1933) developed a colorimetric method for the estimation of the bromide content of body fluids based on the production of a reddish-brown solution of auric bromide when auric chloride and bromide solutions are mixed. More recently Stenger and Kolthoff (1935) have described a colorimetric method based on the fact that phenol red, with a colour change from yellow to red over the pH interval 6.4 to 8.0, reacts with very dilute hypobromite in weakly alkaline solution to form an indicator of the brom-phenol blue type which changes from yellow to blue-violet over the pH range 3.2 to 4.6. Concentrations of 0 to 4000 γ of bromine, present as bromide, can be estimated with an accuracy of 15 to 20%; the method is not applicable to solutions containing over 18,000 γ, since in concentrations from 18,000 to 30,000 γ nearly constant color is produced, while above 30,000 the indicator is attacked and fades.

Experimental

Since the quantity of potassium bromate present in treated wheat flours rarely exceeds 0.005% which corresponds to 0.00239% bromine, microchemical methods must be employed for its estimation. Meyer's (1931) macro-volumetric method for the determination of bromates in flour improvers is obviously unsuited for their estimation in treated flours.

Experiments with Kulman's (1934, 1934a) flotation method for separating the added bromate from the flour showed it to be quite unreliable, particularly for concentrations below 0.003%, the results being uniformly low with unsatisfactory replicability. The colorimetric methods of Scott (1925) employing magenta reagent and of Tod (1933), which is based on the production of auric bromide, were next investigated and abandoned. With the magenta test, difficulty was experienced with adsorption of the violet colour of the chloroform layer at the chloroform-water interface. The Tod method gave low and variable results. As Stenger and Kolthoff (1935) claim only a relatively low accuracy for their method, it was not studied.

The methods available, therefore, resolved themselves into those involving the micro-determination of bromine which require the destruction of the organic material and the quantitative separation of bromine from chlorine and iodine. A survey of the literature indicated that the latter could be satisfactorily accomplished by the partial oxidation procedure outlined by Yates (1933). Preliminary experiments showed this to be the case and the method finally adopted for this and subsequent stages of the determination differed from that of Yates in only a few details.

The reaction apparatus employed by Yates consisted of a 100 c.c. conical flask fitted with a two-holed rubber stopper, the air inlet tube being carried down to the bottom of the flask to ensure maximum aeration. The reaction flask is connected to an absorber consisting of a test tube (10 × 1 cm.) fitted with a two-holed stopper carrying an exit and an inlet tube drawn out to a fine capillary, which extends to within 2 mm. from the bottom of the test tube; only one absorber is used but after aeration for two hours the tube is renewed and the aeration continued for a further three hours. Using a similar set-up and working with potassium bromide solutions of known concentration, it was found that from 4 to 5% greater recovery could be obtained by the addition of a second absorption tube. With this modification, the recovery of bromine present in amounts corresponding to those in treated flours was in the order of 95 to 96%, which is very satisfactory for work of this kind.

However, Francis and Harvey (1933) emphasized the importance of keeping the apparatus free from minute traces of organic matter and specifically warned against the use of rubber joints. They employed an all-glass apparatus comprising a reaction vessel and two small absorption tubes, the ground glass joints being lubricated with distilled water only. In view of their experience, it appeared that higher recoveries and better replicability might be possible with an all-glass apparatus so designed as to eliminate as many connections as possible and to permit of the titrations being carried out directly in the apparatus. Accordingly, at the request of the authors, a special Pyrex glass reaction and absorption vessel with standard taper ground joints was designed and constructed by Binnington (1937) [3] for this purpose, the constructional details of which are shown in Figure 1. In the analysis of potassium bromide solutions the use of this apparatus gave recoveries between 98 and 100% with relatively low variability between replicates.

These results on potassium bromide solutions showed that the modified Yates oxidation and recovery procedures were eminently

[3] D. S. Binnington.

satisfactory and attention was next directed to the determination of potassium bromate in wheat flours, which first involves its separation from the large bulk of organic matter. For the destruction of organic matter in the micro-determination of bromine in blood, Francis and

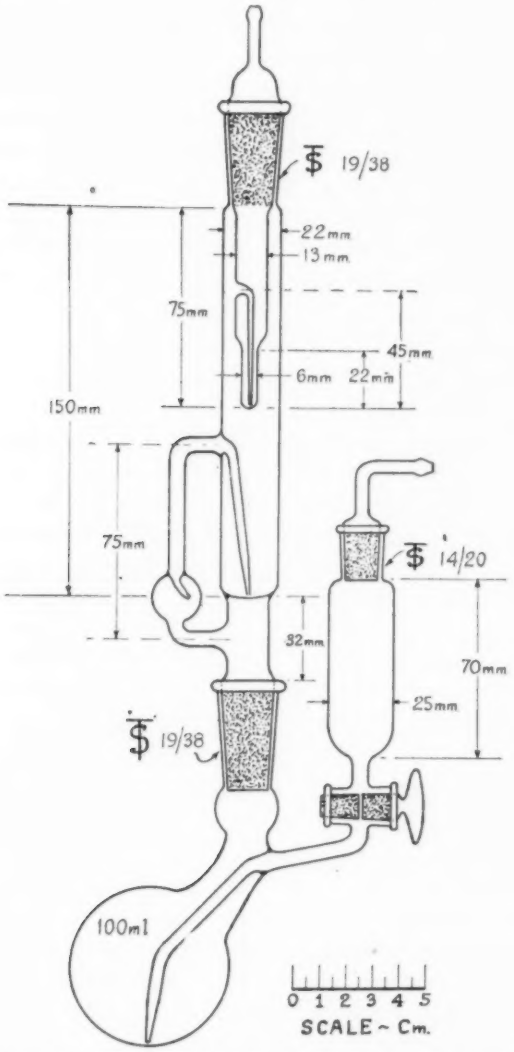

Figure 1. Constructional details of Pyrex glass reaction vessel.

Harvey (1933) add a definite quantity of potassium hydroxide solution and incinerate first in a nickel crucible and finally in a platinum one at 480° to 500° C. After the preliminary ignition the carbonized mass is digested with water on a steam bath, the carbon separated by

filtration, which is again incinerated, digested and filtered. The two filtrates are combined, evaporated and incinerated in platinum.

A series of bromated flours containing 1, 2, 3 and 5 mg. of $KBrO_3$ per 100 g. of flour was prepared and the bromine content determined on a 20-g. sample by the modified Yates method after ashing by a procedure similar to that outlined by Francis and Harvey (1933). The percentage recovery varied from 78 to 92%, the highest value being obtained with the samples containing 0.005% $KBrO_3$. Several modifications were tried without material improvement. The complete destruction of such large quantities of organic matter by this process is difficult and tedious and, in view of the low results, it was decided to try leaching the soluble bromate from the main flour mass before ashing. This resulted in a satisfactory recovery, and the complete method as finally developed follows.

Method in Detail

Apparatus necessary

300 c.c. wide-mouthed, glass-stoppered bottles.
Mechanical shaker.
Centrifuge fitted with head for 100 ml. tubes and capable of being operated at 2,800 r.p.m.
100 c.c. glass centrifuge tubes.
Evaporating dish, capacity 300 c.c., preferably platinum.
Nickel or preferably platinum crucible with lip, capacity 75 c.c.
Mohr pipettes, graduated in 0.10 ml.
Binnington all-glass micro-reaction and absorbing vessel (Figure 1).
Micro-burette, capacity 5 ml.

Reagents

Potassium hydroxide solution, 10%.
Potassium nitrate solution, 0.1%.
Sulphuric acid—concentrated (C.P.).
Chromic-sulphuric acid mixture. Dissolve 20 g. chromic oxide (C.P.) in 120 c.c. distilled water and slowly add, with cooling, 40 c.c. concentrated sulphuric acid (C.P.).
Potassium iodide solution, 10%; freshly prepared.
Starch solution, 0.5%.
Sodium thiosulphate solution, 0.001N; prepared by dilution of 0.1N thiosulphate solution, which has been adequately aged.

Determination

Extraction: Weigh a 20-g. sample into a 300 c.c. wide-mouthed, glass-stoppered bottle, add 75 c.c. of freshly distilled water and stir thoroughly with glass rod to assist complete suspension, setting aside rod for further use. Add a few glass beads and shake for one hour in mechanical shaker. Carefully transfer suspension to a 100 c.c. centrifuge tube; centrifuge for approximately 15 minutes at 2,800 r.p.m. Filter supernatant solution through a filter paper, Watman No. 4, 11 cm., and collect filtrate in a 300 c.c. evaporating dish, preferably of platinum.

By means of the glass rod previously used, break up remaining dough mass in centrifuge tube with 40 c.c. of distilled water; transfer to the wide-mouthed bottle used in the first operation, by means of approximately 35 c.c. distilled water. Great care should be taken that a proper dispersion of the mass is obtained.

Shake for one hour and centrifuge as previously described, filter through the same paper used for the first filtration, wash with approximately 20 c.c. boiling distilled water, combining washings and filtrate in the 300 c.c. evaporating dish.

Add 2.5 c.c. of 10% KOH solution and evaporate on a steam bath to a volume of approximately 30 c.c. Transfer to a lipped nickel or platinum crucible (75 c.c. capacity), rinse evaporating dish thoroughly with boiling distilled water (the washings not to exceed 25 c.c. are collected in the crucible) and evaporate contents of crucible to dryness.

Ashing: Incinerate the crucible and its contents at 500° C. for 30 minutes and cool; break up the carbonized mass by means of a glass rod which is rinsed with approximately 10 c.c. of distilled water, collecting the rinsings in the crucible. Evaporate to dryness and again incinerate at 500° C. for 1½ hours.

If all the carbonaceous matter has not been destroyed, repeat the above procedure and if after the third incineration carbon is still present, moisten with 2 c.c. of a 0.1% KNO_3 solution, evaporate to dryness and ignite at 500° C. until completely ashed.

Oxidation, aeration and titration: The residue is dissolved and transferred to the reaction flask by means of 7 c.c. of distilled water (± 0.1 c.c.) using a 3 c.c. and two 2 c.c. portions for this process. Place 3 c.c. of 10% KI solution and 4 drops of starch solution in the main absorption tube, 1 c.c. of KI solution and 2 drops of starch solution in the secondary trap and connect to the reaction flask.

Partially immerse reaction flask in an ice-water bath and by means of the side-tap funnel carefully and slowly add 2.5 c.c. of concentrated H_2SO_4, taking at least 10 minutes for this addition. To the cold solution in the reaction flask add rapidly by means of the tap funnel 4 c.c. of the chromic acid-sulphuric acid mixture. Air, aspirated through 25% KOH solution, is drawn through the reaction flask by means of a water pump at a rate of approximately one bubble per second. After three hours' aeration, during which period the apparatus is held at room temperature, disconnect the absorption unit, loosen the upper stopper, blow the contents of the trap into the main vessel and wash with two successive 1 c.c. portions of the 10% KI solution. Titration is carried out in the main absorber with 0.001N sodium thiosulphate solution. Wash and dry the absorption unit, recharge the absorbers, and continue the aeration for a further 2 to 2½ hours, after which period titrate the liberated iodine as before.

1 c.c. 0.001N thiosulphate = 0.0000799 gram bromine or 0.0001670 gram $KBrO_3$. To calculate the percentage of $KBrO_3$, multiply the thiosulphate titration in c.c. by 0.000835.

Discussion

It is essential that the evaporated extract be entirely free from moisture before ashing; otherwise trouble is experienced from decrepitation.

The ash should be finely divided before lixiviation and must be perfectly free from carbon. Traces of organic matter seriously influence the results.

In carrying out the oxidation, both direct sunlight and darkness must be avoided.

Scrupulous cleanliness and attention to detail are essential to secure reliable results. The volume of the titer must not be increased by washing down the sides of the absorbing vessel during titration; otherwise low results are secured.

Blank tests should be periodically conducted.

A single determination requires approximately eight hours and one analyst can conduct up to four determinations simultaneously.

Accuracy of the Method

The prepared flours containing 1, 2, 3 and 5 mg. of $KBrO_3$ per 100 of flour were analyzed in duplicate by the above procedure and the mean values obtained were 93.6, 94.8, 92.6 and 97.2%, respectively, of the theoretical. While the recovery of bromine is approximately 3 to 7% below the theoretical, this error is comparatively low for work of this nature where the quantities to be determined are very small.

The precision of the test is indicated in Table I which shows the results of determinations on nine commerical samples of unknown bromate concentration.

Since the standard error of the mean of two determinations equals the standard error of a single determination divided by $\sqrt{2}$, the standard error of the difference between the means of two sets of duplicate determinations equals the standard error (S.E./$\sqrt{2} \times \sqrt{2}$). Taking the level of significance as twice the standard error, a difference of 0.00019% $KBrO_3$ between the means of any two duplicate determinations is statistically significant.

The method is applicable in the presence of chlorates, iodates and persulphates. In the ashing, persulphates are decomposed while chlorates, bromates and iodates are reduced to the corresponding halides. By controlled oxidation with the chromic-sulphuric acid mixture only bromine is liberated. The specificity of the method is shown by the results given in Table II.

TABLE I

RESULTS OF DUPLICATE DETERMINATIONS ON COMMERCIAL SAMPLES

Reference number	Potassium bromate content	
	Mean	Difference between duplicates
	%	%
1	0.001682	0.000225
2	0.001274	0.000072
3	0.001562	0.000047
4	0.000914	0.000228
5	0.001114	0.000141
6	0.001582	0.000176
7	0.001694	0.000047
8	0.001569	0.000068
9	0.001703	0.000017
Mean	0.001455	0.000113

Standard error of single determination 0.000097

TABLE II

EFFECT OF PRESENCE OF OTHER FLOUR IMPROVERS

Sample number	Improvers present	Concentration present	Concentration found as $KBrO_3$	Recovery
		%	%	%
1	Potassium bromate	0.004	0.003930	98.2
2	Potassium bromate	0.004	0.003815	95.4
	Potassium iodate	0.001		
3	Potassium bromate	0.004	0.003825	95.6
	Ammonium persulphate	0.002		
4	Potassium bromate	0.002	0.002020	101.0
	Potassium chlorate	0.002		
5	Potassium bromate	0.002	0.001935	96.8
	Potassium iodate	0.001		
	Ammonium persulphate	0.002		
	Potassium chlorate	0.002		

Summary

Experiments with Kulman's flotation method for separating added potassium bromate from treated wheat flours showed it to be quite unreliable, particularly for concentrations below 0.003%, the results being uniformly low with unsatisfactory replicability. Two colorimetric methods for the estimation of bromine described in the literature were also found to be unsatisfactory.

A method described by Yates for the micro-determination of bromine in blood, which involves ashing in the presence of potassium hydroxide and selective oxidation of the mixed halides with a chromic-sulphuric acid mixture of such concentration that bromide is completely oxidized to bromine, while the chloride remains unaffected and any iodide present is converted to iodate, was found to give good recoveries with potassium bromide solutions. The recovery was improved by

employing a special all-glass micro-reaction and absorbing vessel in which an additional potassium iodide absorber is provided.

Employing these modifications and working with bromated flours of known bromine content, leaching of the added bromate from the flours gives better recoveries than direct ashing.

The method, as finally developed for the estimation of bromine in bromated flours, is described. This involves leaching a 20-g. sample with water, ashing with KOH, selective oxidation of the bromide to bromine with chromic-sulphuric acid mixture in a special reaction and absorbing vessel, aeration and absorption of the bromine in potassium iodide solution and titration with 0.001N thiosulphate.

With flours containing 0.001% to 0.005% $KBrO_3$ the recoveries ranged from 93% to 97%. For a series of nine random duplicate determinations, the difference between duplicates varied from 0.000017% to 0.000228% $KBrO_3$ with a mean of 0.000113.

A difference of 0.000194% $KBrO_3$ between the means of duplicate determinations is statistically significant.

The results are unaffected by the presence of chlorates, iodates and persulphates in the treated flours.

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STUDIES ON THE BAKING QUALITY OF FLOUR AS AFFECTED BY CERTAIN ENZYME ACTIONS. V. FURTHER STUDIES CONCERNING POTASSIUM BROMATE AND ENZYME ACTIVITY

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In a preceding paper (Read and Haas, 1937), we have reported the results of our studies dealing with the action of bromate toward certain proteases. The data presented in that paper did not provide any basis for concluding that bromate inhibits the action of the proteinase of wheat flour. Neither did bromate show any inhibitive action toward pepsin and trypsin or toward the protease contained in Taka diastase, but the activity of papain and bromelin was repressed. In this paper we shall present some further studies concerned with the same problem.

Brief Review of Literature

Reference has already been made in our preceding paper to some of the more important studies and these need not be reconsidered except in two or three instances.

Gortner and Sharp (1923) and Sharp, Gortner, and Johnson (1923) reported detailed studies of some of the colloidal properties of wheat flour as related to flour strength, and reached the conclusion that gluten quality is determined primarily by the colloidal condition of the gluten gel. Gortner (1927) found that the peptizable protein varied inversely with loaf volume. Harris (1932) studied the relation between total protein, peptizable protein and loaf volume. He obtained a high positive correlation between non-extracted protein and loaf volume.

Concerning processes involved in bread manufacture, Gortner (1931) stated that "the salts which are added play a role in the hydration of the gluten colloids and probably tend to tighten the gluten and increase its gas holding capacity. It is a matter of common knowledge among bakers that a saturated solution of calcium sulfate will 'tighten up' the gluten of a weak flour. Certain of the flour improvers which are upon the market probably owe their beneficial properties, at least in part, to their action upon the gluten colloids, in that they

increase the tenacity with which gluten particle adheres to gluten particle in the dough gel."

Gortner, Hoffman, and Sinclair (1929) concluded from a study of 12 wheat flours that there is great variability in the amount of protein that can be extracted from a given wheat flour by various salt solutions of equivalent ionic concentration. There is also an equally striking variability of the proteins of individual flours toward a single salt solution. They believed these differences to be associated with the colloidal properties of the wheat proteins, which may also vary because of heritable differences and environmental conditions.

James and Huber (1927) found that oxidizing agents greatly increased the limit of the extensibility and breaking strength of gluten. Rich (1934) concluded that the effect of artificial maturation and the action of "gluten improvers" such as bromate, must be of a colloidal nature.

Frey and Landis (1932) reported that sulfur dioxide, when present in extremely small amounts, produced a very marked effect on the colloidal properties of gluten. In connection with this statement Alexander (1932) pointed out that it is well known in the paper and the corn products industries that SO_2 exerts marked swelling and disintegrative action on protein-carbohydrate complexes.

Balls and Hale (1936) stated that "cyanides and sulphites, like cysteine and glutathione, are strong reducing agents. They are well-known activators of proteinases of the papain type. This activation results from the reduction of disulphide linkages in the enzyme to sulphydryl groups."

Experimental

The investigative studies to be presented in this paper will deal with the relation of bromic acid to pH, the adsorption of bromate by activated and inactivated enzymic preparations, the effect of sulphite on gluten, the saccharogenic activity of flour as influenced by the bromate and persulphate of potassium, the heat required to destroy the proteinase of malt, and the repressive action of an aqueous extract of soya bean on the activity of certain proteases. The technic employed has been sufficiently described in the preceding paper of this series. (Read and Haas, 1937.)

Relation of pH to Free Bromic Acid

The suggestion of Kosmin (1934) that bromic acid may be slowly set free in the dough batch and thus attack the proteinase contained in the flour would seem to be rather improbable. Bromic acid is a strong oxidizing agent and may be titrated iodometrically. It was considered

advisable to determine what pH is required in order to free bromic acid from bromate. The results of these tests have been recorded in Table I from which it will be noted that the hydrogen-ion concentration necessary for the presence of bromic acid is never reached in a normal dough.

TABLE I

BROMIC ACID FROM BROMATE AS RELATED TO pH

Citrate—HCl buffer mixtures were used to prepare 5% gelatin solutions of varying pH. Temperature maintained at 30° C. in closed cabinet

pH	Titration after 4 hours	Additional titrations for the following 16 hours	Percentage of $KBrO_3$ titratable over a 20-hour period
	C.c. of standard thiosulphate		
Control	7.00	0.00	100
1.9	7.00	0.00	100
2.9	1.40	2.40	54
3.5	0.35	0.90	18
3.9	0.05	0.30	5
4.5	0.00	0.00	None
4.9	0.00	0.00	None

With this information in mind, it was decided to titrate bromate against enzyme preparations before and after the destruction of protease, with a view to ascertaining to what extent the content of bromate might be affected by the presence of active protease. If the variation in titration for the active and the inactive enzymic preparations was definite and of recognizable magnitude, the destruction of enzyme by bromate might be indicated. These data are given in Table II. Here again the results appear to fall in line with those already

TABLE II

BROMATE ADSORPTION BY ENZYME PREPARATIONS

One hundred cubic centimeters of each enzyme preparation were digested with 50 mg. of bromate for 2 hours at 35 C. Then 25 c.c. of each preparation were titrated against 0.05 N thiosulphate.

Preparation	Distilled water controls	Protease active	Protease killed
	C.c. standard thiosulphate		
Merck's diastase (10% extract)	9.15	9.0	9.0
Raw pineapple juice [1]	9.15	8.4	8.7
Papain [1] (6% extract)	9.15	8.4	8.4
Taka diastase (8% solution)	9.15	9.15	9.15
Pepsin (6% solution)	9.15	8.9	8.9
Trypsin (5% solution)	9.15	9.1	9.1

[1] Significant values appear to be indicated by the loss in bromate caused by papain and pineapple uice.

TABLE III

EFFECT OF SODIUM BISULPHITE ON GUM GLUTEN (HUNGARIAN MANUFACTURE) AS MEASURED BY WATER SOLUBLE NITROGEN ACCORDING TO THE PROCEDURE OF JØRGENSEN FOR FLOUR. GLUTEN SAMPLES CONSISTED OF 25 G. EACH

Product	Control (autolytic)	Digested with 10 mg. of $NaHSO_3$	Digested with 20 mg. of $NaHSO_3$
	Mg. soluble nitrogen		
Gum gluten as received	5.2	6.5	6.7
Same gluten heated in water suspension for 30 minutes at 90° C. to destroy protease	5.0	6.9	7.4

TABLE IV

HEAT REQUIRED TO DESTROY PROTEINASE IN MERCK'S DIASTASE OF MALT IN DRY STATE

Amount added	Temperature	Controls unheated	6 hours at 110° C.	12 hours at 110° C.	12 hours at 110° C. 1 hour at 145° C.
Mg.	° *C.*	*Consistency of gelatin after 24 hr. at 27° C.*			
15	25	Very viscous liquid	Very viscous liquid	Very viscous liquid	Firm
	0	Hard	Hard	Hard	Hard
20	25	Viscous liquid	Viscous liquid	Viscous liquid	Firm
	0	Medium	Medium	Hard	Hard
30	25	Liquid	Liquid	Viscous liquid	Medium
	0	Soft	Soft	Medium	Hard
40	25	Liquid	Liquid	Liquid	Soft
	0	Very viscous liquid	Very viscous liquid	Soft	Hard
50	25	Liquid	Liquid	Liquid	Mobile
	0	Viscous liquid	Viscous liquid	Very soft	Hard
60	25	Liquid	Liquid	Liquid	Mobile
	0	Viscous liquid	Viscous liquid	Very viscous liquid	Hard
80	25	Liquid	Liquid	Liquid	Very viscous liquid
	0	Liquid	Liquid	Viscous liquid	Hard

TABLE V

PROTEOLYTIC ACTIVITY AS AFFECTED BY A 10% AQUEOUS EXTRACT OF SOYA BEAN FLOUR[1]

Quantity enzymic preparation added	Temperature	Papain (8% extract)		Raw pineapple juice	
		Controls	2 c.c. of soya bean extract	Controls	2 c.c. of soya bean extract
Drops	° C.	*Consistency of gelatin after 24 hours digestion at 27° C.*			
2	25	Liquid	Liquid	Liquid	Liquid
	0	Hard	Medium	Hard	Fairly hard
3	25	Liquid	Liquid	Liquid	Liquid
	0	Medium	Mobile, soft	Fairly hard	Medium
4	25	Liquid	Liquid	Liquid	Liquid
	0	Mobile, stiff	Viscous liquid	Medium	Soft
5	25	Liquid	Liquid	Liquid	Liquid
	0	Mobile, soft	Viscous liquid	Mobile	Mobile, soft
6	25	Liquid	Liquid	Liquid	Liquid
	0	Very viscous liquid	Liquid	Very viscous liquid	Viscous liquid

[1] An aqueous extract of soya bean contains a powerful fat per-oxidizing enzyme.

obtained by other methods of approach. The loss of titratable bromic acid is to be attributed apparently to its adsorption by, or to its oxidation of organic material other than protease, except possibly for papain and bromelin.

Action of Sodium Bisulphite on Gluten

The marked disintegrating action of sodium bisulphite on gluten is indicated by the results recorded in Table III. (See also Table II, paper IV of this series.) When investigated according to the procedure employed by Jørgensen for the estimation of water soluble nitrogen, the presence of the bisulphite increased the soluble nitrogen, and did so in some proportion to the quantity of salt present. One would thus be induced to conclude that sodium bisulphite is an activator for proteases. On the other hand, the data concerned with gelatin liquefaction would lead to just the opposite conclusion. Our results indicate definitely that the disintegration of gluten by means of the bisulphite is not to be attributed to the activation of protease.

Heat Destruction of Protease Activity in the Dry State

These data have been presented in Table IV. The enzymic product used was Merck's diastase of malt (U. S. P.—IX). The high proteolytic activity of this product makes it a suitable agent for study. In the dry state the proteinase of malt is shown to be surprisingly resistant to heat. This would indicate that the proteinase in flour would likewise be very stable towards heat.

Effects of an Aqueous Extract of Soya Bean Flour on Gelatin Proteolysis

The soya bean contains a very potent fat-peroxidizing enzyme which is water soluble. Some liquefaction tests were made with a 10% extract of soya bean flour to ascertain if such an extract contained any factor or factors which might affect the activity of several proteases. The results have been recorded in Tables V and VI. It is interesting to note that with the exception of papain and bromelin,

TABLE VI

PROTEOLYTIC ACTIVITY AS AFFECTED BY A 10% AQUEOUS EXTRACT OF SOYA BEAN FLOUR [1]

Quantity enzymic preparation added	Temperature	Taka diastase (5% solution)		Trypsin (1% solution)	
		Controls	2 c.c. of soya bean extract	Controls	2 c.c. of soya bean extract
Drops	° *C.*	*Consistency of gelatin after 24 hours digestion at 27° C.*			
2	25	Liquid	Liquid	Liquid	Very soft
	0	Very soft	Medium	Soft	Hard
3	25	Liquid	Liquid	Liquid	Mobile
	0	Very viscous liquid	Medium	Very viscous liquid	Hard
4	25	Liquid	Liquid	Liquid	Mobile, soft
	0	Viscous liquid	Soft	Viscous liquid	Hard
5	25	Liquid	Liquid	Liquid	Mobile, very soft
	0	Liquid	Very viscous liquid	Liquid	Hard
6	25	Liquid	Liquid	Liquid	Very viscous liquid
	0	Liquid	Very viscous liquid	Liquid	Hard

[1] An aqueous extract of soya bean contains a powerful fat per-oxidizing enzyme.

the proteases investigated were repressed in their activity on gelatin. It may be added that Merck's diastase of malt was also inhibited to about the same extent as was pepsin. Trypsin appeared to be the most affected. It will be recalled from our preceding paper (1937) that in the case of bromate, papain and bromelin were the only proteases which were adversely affected, while in this instance they were the only proteases which were not adversely affected by the extract of soya bean.

Bromate and Diastasis

On the two flours investigated (Table VII) the effect of bromate was to increase diastasis. The results were consistently definite in this

TABLE VII

SACCHAROGENIC ACTIVITY AS INFLUENCED BY THE BROMATE AND PERSULFATE OF POTASSIUM

Dosage per 5 g. of flour	Flour No. 153956	Flour No. 150004
Mg.	*Mg. of anhydrous maltose per 10 g. of flour*	
Controls (autolytic)	318	416
0.2, $KBrO_3$	322	434
0.5, $KBrO_3$	335	447
1.0, $KBrO_3$	335	449
2.0, $KBrO_3$	340	452
4.0, $KBrO_3$	358	443
5.0, $K_2S_2O_8$	321	—
10.0, $K_2S_2O_8$	340	—

TABLE VIII

EFFECTS OF CERTAIN CHEMICAL TREATMENTS ON PROTEOLYTIC ACTIVITY [1]
Merck's Diastase of Malt (10% aqueous extract)

Amount of extract added	Temperature	Controls	Quantity of chemical agents shown in milligrams					
			5 $NaVO_3$	10 $NaVO_3$	Controls pH, 1.9	5 $BKrO_3$ pH, 1.9	Controls pH, 2.9	5 $KBrO_3$ pH, 2.9
Drops	° *C.*		*Consistency of gelatin after 24 hours digestion at 27° C.*					
1	25	Liquid	Liquid	Liquid	Thick liquid	Thick liquid	Thick liquid	Thick liquid
	0	Soft	Soft	Soft	Hard	Hard	Hard	Hard
2	25	Liquid	Liquid	Liquid	Thick liquid	Thick liquid	Thick liquid	Thick liquid
	0	Mobile	Viscous liquid	Viscous liquid	Hard	Hard	Hard	Hard
3	25	Liquid	Liquid	Liquid	Thick liquid	Thick liquid	Thick liquid	Thick liquid
	0	Viscous liquid	Liquid	Liquid	Hard	Hard	Hard	Hard
4	25	Liquid	Liquid	Liquid	Thick liquid	Thick liquid	Thick liquid	Thick liquid
	0	Liquid	Liquid	Liquid	Hard	Hard	Hard	Hard
5	25	Liquid	Liquid	Liquid	Very viscous liquid	Very viscous liquid	Very viscous liquid	Very viscous liquid
	0	Liquid	Liquid	Liquid	Hard	Hard	Hard	Hard

[1] It will be observed from this table that sodium meta-vanadate does not inhibit the activity of malt proteinase on gelatin, also that hydrogen-ion concentration of pH − 1.9 and pH − 2.9 practically prevents any hydrolysis of the gelatin.

direction, but were not always consistently uniform. It is probable that the stimulative action is not a direct one.

Influence of pH and Sodium Meta-Vanadate on Proteolysis

From Tables VIII and IX it will be observed that sodium meta-vanadate is not inhibitory toward the proteinase of malt or toward the proteinase of autolyzed yeast, also that enzyme activity is practically prevented by pH 1.9 and pH 2.9.

TABLE IX

EFFECTS OF CERTAIN CHEMICAL TREATMENTS ON THE PROTEOLYTIC ACTIVITY OF AUTOLYZED YEAST [1]

Quantity of enzymic preparation added	Temperature	Mg. of chemical agent added to each flask			
		Enzymic controls	10 $KBrO_3$	10 $NaVO_3$	10 $NaHSO_3$
Drops	° C.	*Consistency of gelatin after 24 hours digestion at 27° C.*			
5	25	Viscous liquid	Viscous liquid	Viscous liquid	Very viscous liquid
	0	Firm	Firm	Firm	Hard
8	25	Liquid	Liquid	Liquid	Viscous liquid
	0	Fairly firm	Fairly firm	Fairly firm	Hard
10	25	Liquid	Liquid	Liquid	Viscous liquid
	0	Fairly soft	Fairly soft	Mobile	Hard
12	25	Liquid	Liquid	Liquid	Liquid
	0	Mobile	Mobile	Mobile, soft	Very firm
15	25	Liquid	Liquid	Liquid	Liquid
	0	Mobile, soft	Mobile, soft	Mobile, very soft	Fairly firm
18	25	Liquid	Liquid	Liquid	Liquid
	0	Very viscous liquid	Very viscous liquid	Viscous liquid	Fairly soft
20	25	Liquid	Liquid	Liquid	Liquid
	0	Viscous liquid	Viscous liquid	Liquid	Soft

[1] To obtain the enzymic preparation, yeast was autolyzed by the method described in British Patent No. 450, 529 (1936). The yeast was first plasmolyzed by the addition of from 5 to 10% of ethyl acetate. Water was then added and the pH adjusted to approximately 6.3 by adding a 5% solution of trisodium phosphate. Autolysis was allowed to proceed for 30 hours at 35° C. during which time the pH was maintained at approximately 6.3 by several separate additions of the phosphate solution. By this procedure, the original weight of yeast had been increased by approximately 150%. Material in suspension was readily removed by centrifuging.

On the basis of the investigative work thus far conducted, it seems to us that the problem of the nature of the action of bromate as a "dough improver" is one which demands further and more detailed study. We are unable to support the conclusions reached by Jørgensen and other investigators, namely, that the ameliorative action of bromate in flour dough is to be attributed to the inhibitory influence of the bromate on the proteinase contained in the flour.

Summary

The data recorded in Table I indicate that the pH required for the production of free bromic acid from bromate never occurs in the dough batch.

The disintegration of gluten by sodium bisulphite cannot be attributed to proteinase activation.

The liquefaction tests which are recorded in Table IV show that the proteinase of malt (in the dry state) is very stable toward high temperatures.

Bromate and persulphate of potassium, when present in suitable amounts, promoted saccharogenic activity.

An aqueous extract of soya bean contained some factor or factors which repressed the activity of trypsin, pepsin and the protease of Taka diastase, but did not inhibit the activity of papain and bromelin. Like trypsin and pepsin the proteinase of malt was also repressed.

Potassium bromate and sodium meta-vanadate exerted no inhibitive action on the protease obtained from autolyzed yeast (Table IX), but sodium bisulphite did inhibit the activity of yeast protease to a considerable degree. This behavior of the bisulphite is contradictory to certain statements which have appeared in recent literature.

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WIDE-RANGE VOLUME-MEASURING APPARATUS FOR BREAD

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Included in the bread scoring method [1] of the American Institute of Baking is the volume of the loaf. The volume of the loaves received for scoring varies from 1,200 c.c. to 3,000 c.c. and the lengths from 8 inches to 14 inches. The determination of volumes over this wide range was a perplexing problem for some time.

Much experimental work has been carried on in order to find a piece of equipment that would measure with a fair degree of accuracy the volumes of loaves of this wide variation.

Preliminary Experiments

Preliminary experiments were carried out using the hour-glass type of device. Several modifications of this type are on the market. This type has been modified and adapted to small loaves by Geddes and Binnington (1928). Malloch and Cook (1930) have modified the design still further in order to increase the ease of construction and the accuracy of operation. Two possibilities of error were noted with this type of device:

First, the error introduced if the loaf is not perfectly flat on the bottom. In most of the hour-glass types described in the literature, the rape seed or material used is not able to get under the loaf, since the loaf rests on the bottom of the chamber which contains it. In some cases this error was found to be of considerable significance, as will be seen later.

Second, the error due to the enormous quantity of material used in the device. The apparatus must be of large enough construction to hold the largest loaf. Thus, it is apparent that when the volume of a small loaf is measured, an unusually large quantity of material must be used. This was found to introduce a considerable error even when the apparatus was recalibrated with a standard loaf of approximately the same volume. This undoubtedly is due to the variations in the degree of packing.

[1] As revised August, 1936.

An apparatus designed about ten years ago by the American Institute of Baking and listed by E. H. Sargent and Company overcomes the first error to some extent, since the loaf is more or less suspended in a large chamber. However, there are other disadvantages, especially the enormous quantity of material required to operate it and the small range of volumes which it will handle.

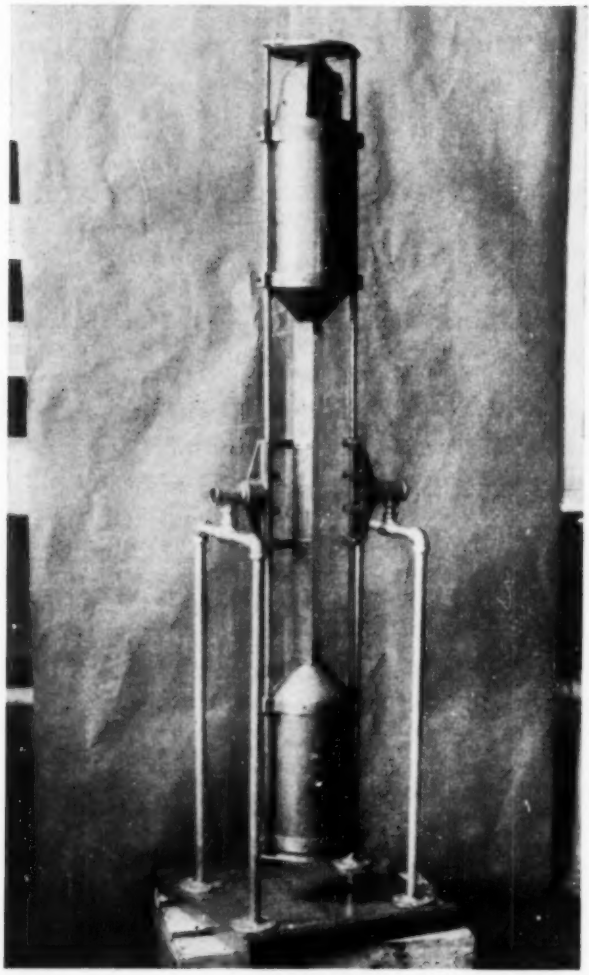

Figure 1*a*. The modified hour-glass type apparatus. The loaf of bread is held above the measuring chamber so that it may be seen.

The first error mentioned was overcome almost completely by a device similar in construction to those described by Geddes and Binnington, and also Malloch and Cook (Figures 1*a* and 1*b*). How-

ever, the method of suspending the loaf is different, it being suspended in the chamber on the thin steel rod *D* by means of the two aluminum washers *B* and the set screw *C*. This allows the material used to fill all of the space around the loaf except that covered by the two washers. This also has the advantage that it will accommodate sliced bread and handle loaves of varying lengths with the minimum amount of seed.

Although the apparatus shown in Figures 1*a* and 1*b* was designed to reduce the quantity of material used, the second error due to the variations in the degree of packing was still as great as ever.

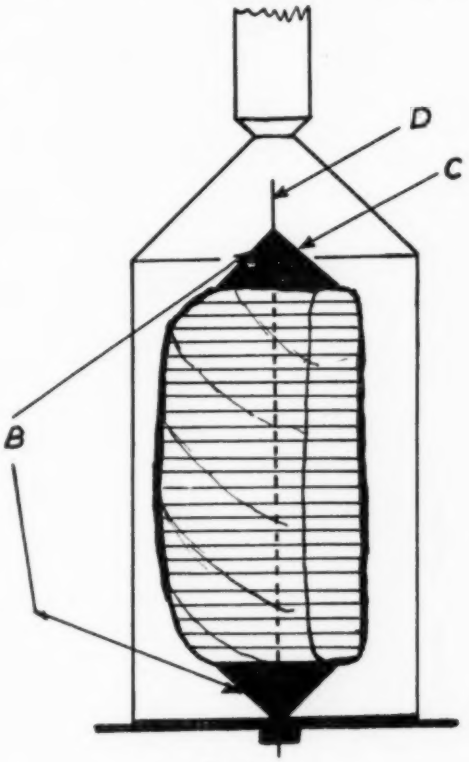

FIG. 1*b*.

In order to overcome the second error, a material was sought which would have practically no degree of packing. Many different materials were tried. Graduated cylinders were used and the degree of packing was measured. All of the seeds tried showed a large degree of packing. Of all the seeds tried, rape seed and rice gave the most reproducible results when allowed to flow freely through an orifice into the cylinder. Rice gave somewhat better checks than rape seed, but was more easily damaged. Static electricity, caused by the flow of the seeds over the

glass, seemed to be an important factor in the case of light materials such as flax seed and rape seed. Attempts to ground the cylinder were helpful but not satisfactory.

Glass beads (1/8 inch diameter at widest point) were better than the seeds, and aluminum balls (1/8 inch diameter) had hardly any degree of packing. However, when glass beads and the aluminum balls were used in the volume apparatus with various standard loaves, the errors were larger than with rape seed. It is felt this is due to the increased weight over that of the rape seed. For example, the error on various determinations of the same loaf was 0.4% for rape seed as compared to 0.8% for glass beads.

Since most inaccuracies enter from the material used, it is obvious that the smaller amount used the less will be the possible error. Thus, the following apparatus is the type that was finally adopted. It has been in use for eight months and has proved entirely satisfactory.

Other investigators (*e.g.*, Whitcomb, 1925) have mentioned trying similar devices; however, their procedure was different from that given below.

Description and Use of the Apparatus

The apparatus is shown in Figure 2 and is simply a box made of 1/2-inch thick plywood. The box is made with inside dimensions of 5 in. × 5 in. × 15 in. Blocks also of plywood were made to fit inside the box in three sizes—5 in. × 5 in. × 1 in., 5 in. × 5 in. × 2 in., and 5 in. × 5 in. × 3 in. The dimensions of the box are accurate to 1/32 of an inch. With these blocks and others, if necessary, it is possible to adjust the volume of the box to almost any desired value. As many of the blocks are used as are necessary to leave just about 1/2 inch space at each end of the loaf. A thin layer (about 1/2 inch) of rape seed (or other material used) is placed on the bottom of the box, then the loaf whose volume is to be measured is placed in the box. Originally, the seed was allowed to flow into the box until full from an overhead hopper, which delivered a constant flow from a uniform height. The same degree of accuracy is accomplished, however, if the seeds are poured into the box through a wire screen with 1/4-inch openings.[2] The excess beads are scraped off the top, level with the box, by the use of a straight edge (the same must be used continually). Then the seeds around the loaf are poured off and weighed to the nearest 1/4 ounce. This weighing introduces an error in most cases of less than 0.25% when glass beads or aluminum balls are used, and of less than 0.5% when rape seed is used.

[2] Experiments with a wire screen of this type and graduated cylinders showed that rape seed and glass beads gave a uniform degree of packing under these conditions.

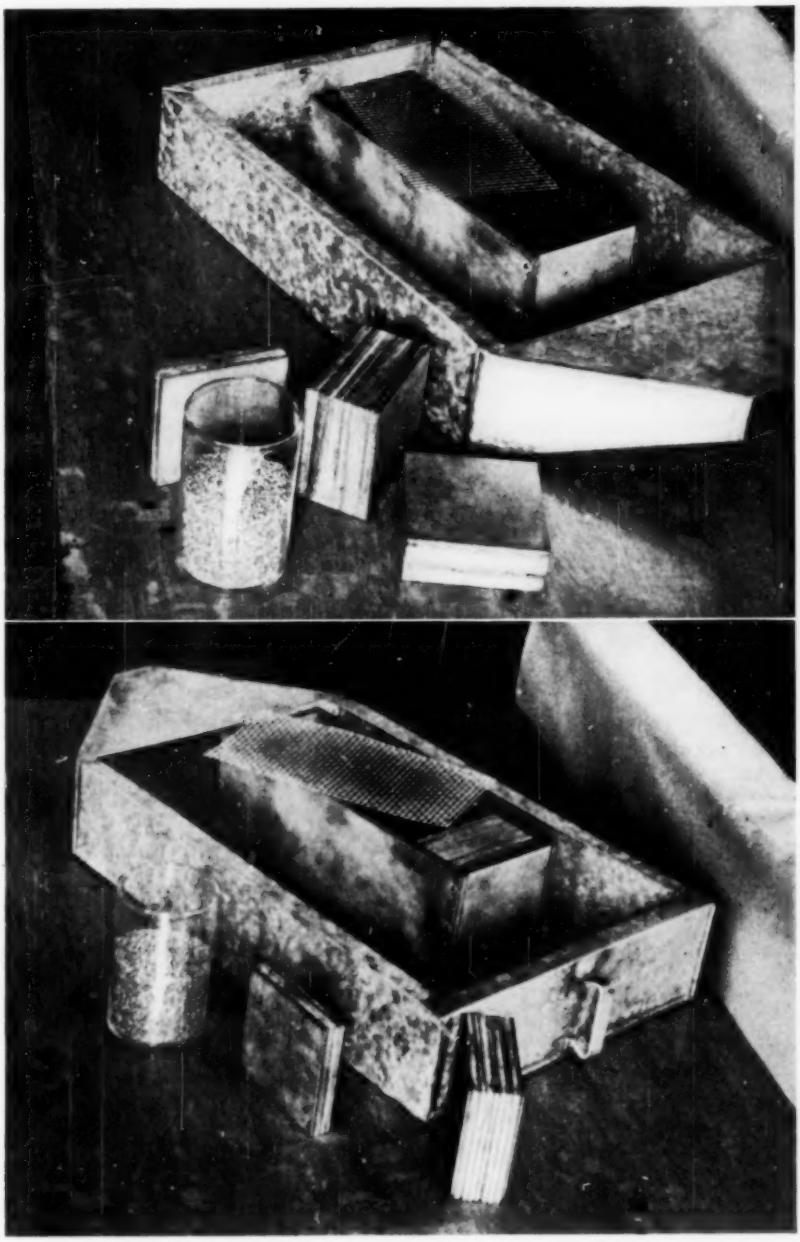

Figure 2. Wide-range-volume-measuring apparatus for bread. The top picture shows the empty box. The bottom picture shows the box with one block in place. The wire screen is set diagonally to show up in the picture. The blocks not in use and some of the beads are shown in both pictures.

By weighing the rape seed or other material used, it is possible to eliminate the large error due to the difference in the degree of packing that occurs in most other set-ups of this type, where the rape seed is measured in a graduated cylinder.

Experiments have shown that seed (or material used) pack differently in an empty box, in a box containing a loaf, and in a graduated cylinder. Where the cylinder is dispensed with and the rape seed weighed rather than measured, and the unit weight of seed determined by calibration using standard loaves, this error is reduced to a minimum. Due to this and tests with objects other than model loaves, the authors agree with Malloch and Cook that the use of water-filled balloons as recommended for calibration of loaf-measuring apparatus by Harrel (1928) is not satisfactory since they do not even remotely approach the shape of a loaf.

At first the arrangement used to catch the overflow seed and the seed from around the loaf was merely a cardboard box set on a slant, with a funnel-shaped end to allow the beads to run out into a can of tared weight. However, we now have a simpler construction from galvanized iron, as shown in Figure 2.

Knowing the volume of the box and the volume of the weight of the seed or other material used (by calibration), the volume of the loaf can be calculated.

For practical purposes the volume of the box can be taken as what the measurements indicate, that is, 5 in. × 5 in. × 15 in. = 375 cubic inches (or less, depending upon the blocks used in the box). The accuracy of the construction of the box is such that the volume taken in this way introduces an error within 1%. It is possible to decrease this error by determining the measurements of the box more accurately.

Calibration of Apparatus

The weight of a unit volume of seed (or material used) was determined by using one standard loaf and varying the volume of the box and by using three standard loaves and keeping the volume of the box constant.

The volumes of the standard loaves were determined by immersion in water. The values are given in Table I.

Loaves 1, 3 and 4 were coated with paraffin, as described by Malloch and Cook. The volumes of the loaves could be determined by this method with an accuracy of 0.1%.

The values in Table II were obtained with the standard loaves 1, 2 and 3, and the same procedure as described above under Description of Apparatus.

TABLE I

VOLUME OF STANDARD LOAVES

(Determined by immersion in water at 25° C.; accuracy 0.1%)

Loaf number	Description	Volume of loaf	
		In c.c.	In cu. in.
1	Bread, paraffin covered	1239	75.4
2	Aluminum model	1922	117.2
3	Bread, paraffin covered	2171	132.3
4	" " "	2393	145.8

TABLE II

CALIBRATION OF MATERIAL

(Using loaves 1, 2 and 3; varying the volume of the box with loaf 2)

A	B	C	D	E	A − B	$\frac{A-B}{E}$	F
Volume box in cubic inches	Volume of standard loaf from Table I in cubic inches	Number of separate determinations	Variation in separate determinations in %	Weight of material to fill box with loaf. Average separate determinations in ounces	Volume of material in cubic inches	Cubic inches per ounce	Averages, cubic inches per ounce
Calibration of glass beads							
253.0	117.2	4	0.36	121.1	135.8	1.121	1.118
276.5	117.2	3	0.25	142.5	159.3	1.118	
301.0	117.2	2	0.18	165.6	183.8	1.110	
326.0	117.2	2	0.09	187.6	208.8	1.113	
276.5	75.4	2	0.27	180.4	201.1	1.113	
276.5	132.3	2	0.35	127.5	144.2	1.132	
Calibration of aluminum balls							
253.0	117.2	4	0.39	132.0	135.8	1.028	1.029
276.5	117.2	2	0.00	156.0	159.3	1.022	
276.5	132.3	2	0.48	139.1	144.2	1.037	
Calibration of rape seed							
253.0	117.2	2	1.49	50.6	135.8	2.68	2.68
276.5	117.2	2	1.49	59.4	159.3	2.68	
301.0	117.2	2	1.49	68.5	183.8	2.68	
350.0	117.2	2	0.56	86.8	232.8	2.68	
276.5	75.4	2	0.00	75.3	201.1	2.67	
276.5	132.3	2	0.37	53.6	144.2	2.69	

Column F of Table II gives the averages of the cubic inches per ounce for the various materials. This factor (cubic inches per ounce) multiplied by the weight of the respective material in ounces will give the corresponding volume.

Throughout this paper the volumes have been expressed in cubic inches. Cubic inches may be converted to cubic centimeters by multiplying by 16.4.

In Figure 3, the weight of material in ounces is plotted against the volume of material in cubic inches from Table II.

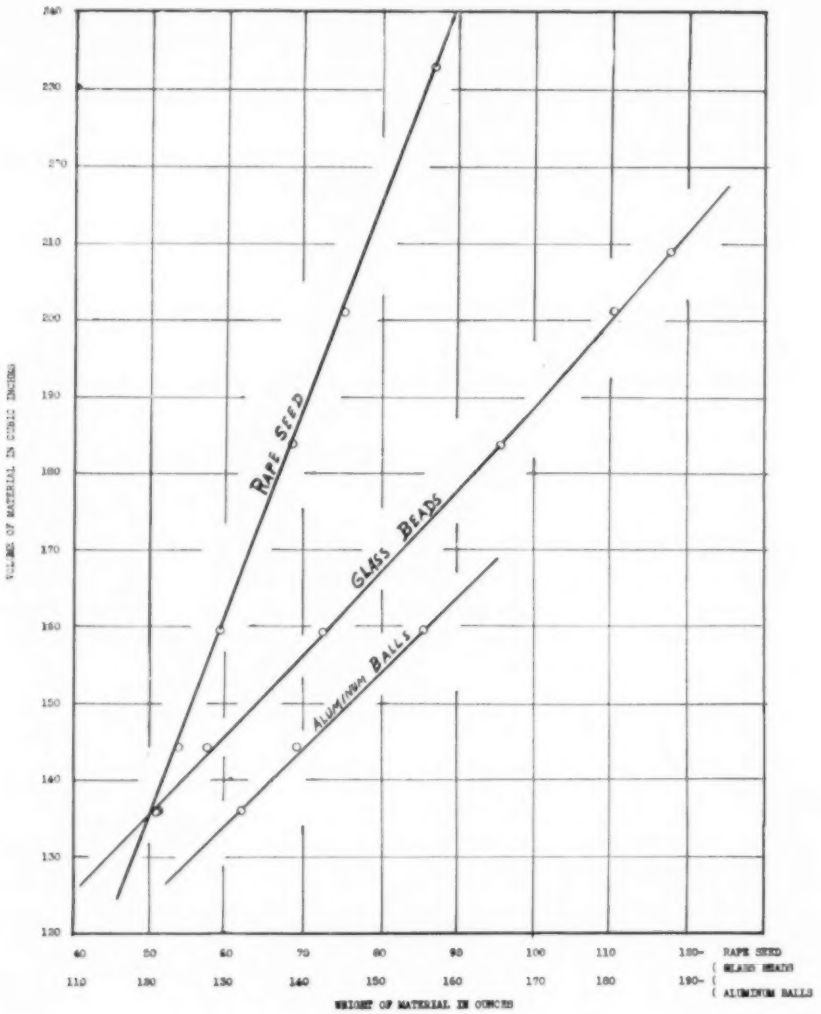

FIG. 3.

The loaf volume can be determined in two ways. First, by using the calibration curves of Figure 3, by means of which the volume of the material corresponding to its weight in ounces can be read directly.

TABLE III

ACCURACY OF THE APPARATUS

(Paraffin-covered loaves, numbers 1, 3 and 4, were used)

A	B	C	D	A – D	E	F
Volume of box in cubic inches	Volume of standard loaf from Table I in cubic inches	Weight of material to fill box with loaf in ounces	Volume of material from Figure 3 in cubic inches	Volume of loaf in cubic inches	Maximum error from volume in B column in %	Average error from volume in B column in %
Using glass beads						
253.0	75.4	160.00	177.9	75.1		
253.0	75.4	159.75	177.7	75.3	0.53	0.27
276.5	75.4	180.50	200.7	75.8		
276.5	75.4	180.75	201.0	75.5		
276.5	132.3	127.75	143.5	133.0		
276.5	132.3	127.25	143.0	133.5	0.91	0.57
301.0	132.3	150.50	167.8	133.2		
301.0	132.3	150.25	167.5	133.5		
301.0	145.8	140.00	156.3	144.7	0.75	0.48
301.0	145.8	139.25	155.5	145.5		
Using aluminum balls						
228.0	75.4	149.25	152.8	75.2	1.06	0.66
228.0	75.4	149.75	153.4	74.6		
276.5	132.3	139.50	143.2	133.3	1.28	1.00
276.5	132.3	138.75	142.5	134.0		
276.5	145.8	126.00	130.0	146.5		
276.5	145.8	125.25	129.3	147.2	1.03	0.75
301.0	145.8	150.50	154.3	146.7		
301.0	145.8	150.00	153.7	147.3		
Using rape seed						
253.0	75.4	66.25	177.8	75.2		
253.0	75.4	66.75	179.3	73.7	2.27	1.23
276.5	75.4	75.25	202.0	74.5		
276.5	75.4	75.25	202.0	74.5		
276.5	132.3	53.75	144.5	132.0		
276.5	132.3	53.50	144.0	132.5	0.98	0.38
301.0	132.3	62.50	168.0	133.0		
301.0	132.3	62.75	168.6	132.4		
301.0	132.3	63.25	170.0	131.0		
276.5	145.8	49.00	132.2	144.3		
276.5	145.8	49.00	132.2	144.3	1.44	1.24
301.0	145.8	58.50	157.3	143.7		
301.0	145.8	58.50	157.3	143.7		

Second, the average factors (cubic inches per ounce) given in column F of Table II may be used.

Accuracy of the Apparatus

In Table III the determinations of the volumes of the three paraffin-covered loaves (1, 3 and 4) are tabulated. The calibration curves given in Figure 3 were used. Loaves 1 and 3 were used in the calibration; however, it will be noted that other volumes of the box were used in this case. Loaf 4 was not used in any way in the calibration.

From Table III glass beads show a smaller percentage error than rape seed. Aluminum balls fall between glass beads and rape seed; however, not enough of them were available to make as many tests as was possible with the glass beads and rape seed. By using glass beads the maximum error is under 1%; however, when two or more determinations are averaged, the error is decreased to about 0.5%.

By recalculating all of the determinations of Table III, using the factors given in column F of Table II, it was found that the maximum error for glass beads was 1.5% and the average error 1%. The corresponding errors for aluminum balls and rape seed were about the same as those found in Table III.

The same tests as those given in Table II were repeated using glass beads only, but with no material having been placed in the box under the loaf. The average percentage error was 0.6 with loaf 1; 3.5 with loaf 3; and 2.2 with loaf 4. Thus, with loaves 3 and 4 large errors are introduced if material is not placed on the bottom of the box before the loaf is placed in it.

Advantages of the Apparatus

The apparatus described is cheap to construct. If it can not be made in the laboratory, a cabinet maker will make the whole thing for about five dollars. The overflow arrangement can be constructed out of galvanized iron by a tinsmith for an additional eight dollars; however, cardboard serves nearly as well. The most expensive item is the glass beads. They cost about seventy five cents per pound, and for the range of volumes with which we have to deal, 15 pounds are necessary. The glass beads, however, are washable; thus, they do not have to be replaced. They stand up very nicely in the apparatus and work well with sliced bread.[3]

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[3] The beads were obtained from the International Importing Bead and Novelty Company, 25 East Washington Street, Chicago, Illinois. When purchased, they were coated with a coloring matter, which must be removed before they are used. This can be accomplished by soaking for 24 hours in a strong soap solution or by means of an organic solvent such as benzene.

The accuracy of the apparatus, using a calibration curve over the range it is being used is less than 1%. If the volumes of the loaves which are to be determined vary only a small amount, the accuracy can be increased. In this case a box without any blocks can be used and the volume of the box determined quite accurately. This decreases the error due to the limit of accuracy in the construction of the box and blocks. The importance of this factor was pointed out by Harrel. This also means that approximately the same quantity of beads will be used all of the time. Experiments have shown that when the box method is used in this way the percentage error is less than with any of the hour-glass types tested.[4]

Summary

An innovation in the hour-glass type of apparatus is made.

A simple and quick apparatus for measuring loaf volume is described. The method of calibration, the way it is used, the accuracy, and the advantages of the apparatus are given.

The relative merits of various materials which can be used in the apparatus are discussed.

Acknowledgment

The authors wish to express their sincere appreciation to the Hubbard Portable Oven Company, Chicago, Illinois, for making the hour-glass type of apparatus; to the Hartford Steel Ball Company, Hartford, Connecticut, for lending the aluminum balls; and to Messrs. Morrill, Norton and Killen of the staff of the American Institute of Baking for helping with the preliminary work.

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[4] A box of any size can be constructed to meet any requirement of loaf volume.

A STUDY OF GLUTEN PROTEIN FRACTIONATION FROM SODIUM SALICYLATE SOLUTION. PART II. BREAD WHEAT GLUTEN FRACTIONATION

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Introduction

In a recent paper the author (Harris, 1937) reported the results of an investigation into the gluten fractions of durum wheat flours, using the sodium salicylate dispersion method, followed by fractional precipitation of the dispersed gluten through the successive additions of concentrated $MgSO_4$ solution, as developed by McCalla and Rose (1935). In this study a significant positive relationship was found between the quantity of protein thrown down by 3 c.c. of the $MgSO_4$ solution and loaf volume. The quantity of wet crude gluten washed from the flours was also positively and significantly correlated with loaf volume, but the multiple correlation computed between the protein fractionated by 3 c.c. $MgSO_4$ and quantity of wet crude gluten with loaf volume was significantly higher than either of the total correlations. A gain of information in regard to loaf volume, or gluten strength, was thus obtained through a knowledge of the protein fractionated. In this instance loaf volume obtained by a short fermentation period of two hours and the incorporation of 5% of sucrose in the baking formula was considered to be a measure of the strength of the flour gluten. The loaf volume obtained by this method yielded a positive correlation of .9594 with the values obtained by the standard fermentation method. The quantities of protein removed by further fractionation did not have a relationship of any great importance with loaf volume. The initial protein fraction was not related to the crude protein content of the flours.

Harris and Bailey (1937) investigated the relation between the three thermal fractions of the gluten proteins employing the method worked out by Blish and Sandstedt (1933). These workers found the final or gliadin fraction to be positively correlated with loaf volume in the instance of 19 samples of *T. vulgare* wheats. The sodium salicylate method, however, did not involve the use of acid, alkali or alcohol which tend to denature protein, and appeared to be less cumbersome and laborious. In order to ascertain whether the fractionation of

gluten proteins from sodium salicylate dispersions by $MgSO_4$ solutions would yield the same information relative to loaf volume in the instance of hard red spring wheats as in the case of durum wheats, the following research was undertaken.

Experimental

Material and Methods

A 70% patent flour was milled from 30 samples of hard red spring wheat of the 1936 crop grown under various conditions of soil and climate. Wet crude gluten was washed from these flours according to the method followed by the author in the previous investigation cited, and recommended by Dill and Alsberg (1924). The washed gluten was dispersed in 10% sodium salicylate solution and fractionated into three portions by successive additions of 3, 8 and 10 c.c. of concentrated $MgSO_4$ solution. A fourth fraction remained in solution, and was not determined owing to difficulties mentioned in the previous paper. The fractions were removed from the residual solution by centrifuging approximately 20 minutes, and the quantity of protein separated determined by a modified form of the Kjeldahl-Gunning procedure. Foaming of the dispersions was prevented by the addition of a few drops of capryl alcohol. Moistures on the flours were determined in the air oven at 135° C. ± 2° for one hour. Protein determinations were made on the wet crude gluten, and the gluten protein of the flour calculated therefrom. The gluten determination is not commonly used as a method for evaluating flour strength, but in the present instance it was necessary to obtain crude gluten for the purposes of fractionation and the extra time and labor involved in determining the gluten quantity and protein content were of no great importance. Ash was determined by the magnesium acetate method advocated by Bailey (1937) using 5 c.c. of acetate solution and an ashing time of one hour at approximately 700° C.

These flours were baked by the standard A. A. C. C. method, using 5% of sugar and a two-minute mix in the Hobart-Swanson. The loaf volume was considered to be a criterion of flour strength in the investigation, and no baking score was calculated for the flours. This attribute is subject to quantitative measurement, no question of individual judgment enters into its evaluation, and in this study the measurement of gluten strength alone was the paramount issue.

A further series of six flours was experimentally milled from a sample of hard red spring wheat, and various separations made corresponding roughly to different millstream flours produced in a commercial mill. The purpose of studying this set of flours was to learn, if possible, the quantity of protein removed as the first fraction by 3 c.c. of

$MgSO_4$ solution from flours of various baking strengths milled from the same wheat sample and presumably containing material originating in different portions of the wheat kernel. Harris (1931) found great variability in protein peptizability among millstream flours and attributed these differences to some extent to variation in the chemical constituents located in distinct parts of the berry. In the present instance only the first protein fraction was determined, as it has been found to be the one principally linked with loaf volume.

Discussion

The crude protein content of the wheat used in this study and the flours milled therefrom are shown in Table I, with ash, gluten content,

TABLE I

COMPARATIVE DATA ON FLOURS AND WHEAT PROTEIN (13.5% MOISTURE BASIS)
Results Arranged in Order of Increasing Loaf Volume

Sample number	Crude protein (N×5.7)		Ash	Wet crude gluten	Protein (N×5.7) in gluten	Gluten protein (N×5.7) in flour	Soluble protein per 100 c.c. solution	Loaf volume
	Wheat	Flour						
	%	%	%	%	%	%	*Mg.*	*C.c.*
37–1–74	16.3	13.9	0.38	40.5	28.7	11.6	436.0	319
81	16.4	14.9	.54	39.2	30.5	12.0	427.5	320
133	17.1	15.6	.53	44.0	28.8	12.7	416.1	342
80	16.3	14.7	.56	38.9	31.0	12.1	444.6	386
137	16.6	15.0	.50	42.5	30.6	13.0	438.9	418
68	16.7	14.1	.38	43.4	28.4	12.3	461.7	433
71	16.4	14.4	.36	44.2	28.5	12.6	521.6	458
77	15.4	13.4	.44	39.2	32.0	12.5	544.4	460
79	14.5	12.6	.50	38.9	29.5	11.5	510.2	469
78	15.4	13.0	.52	39.3	30.9	12.1	513.0	470
76	16.1	13.9	.39	41.6	30.7	12.8	538.7	472
72	19.5	16.6	.35	58.0	23.9	13.9	433.2	485
83	16.4	15.0	.50	43.1	30.3	13.1	481.7	488
73	16.8	14.8	.42	43.9	29.1	12.8	476.0	498
75	16.8	14.3	.27	48.2	27.8	13.4	478.8	500
62	18.2	15.6	.43	50.1	26.6	13.3	501.6	508
135	16.5	14.9	.40	41.4	30.5	12.6	461.7	515
64	16.9	14.9	.43	46.4	30.3	14.0	550.0	524
69	17.2	14.7	.36	44.5	29.2	13.0	644.1	534
65	17.5	15.4	.45	49.0	26.3	12.9	510.2	543
70	17.5	14.9	.52	47.7	28.9	13.8	541.5	544
115	17.4	15.3	.38	46.6	29.0	13.5	487.4	560
95	18.6	16.9	.50	52.2	28.1	14.7	473.1	575
37–2–9	15.8	13.3	.33	46.5	26.4	12.3	518.7	599
37–1–109	18.3	16.1	.42	48.6	29.1	14.1	487.4	610
37–2–3	16.7	14.1	.34	48.4	26.3	12.7	513.0	615
37–1–101	18.5	17.0	.54	52.3	27.8	14.5	478.8	616
37–2–2	16.1	13.5	.35	48.7	26.8	13.0	495.9	622
11	16.4	14.6	.37	49.9	27.5	13.7	521.6	670
10	16.4	14.0	.34	50.8	25.2	12.8	507.3	691

protein soluble in 10% sodium salicylate solution, and loaf volume. These values are arranged in order of increasing loaf volume, and cover a range of 372 c.c. from the smallest to the largest loaf. There does not appear to be any trend toward increase in wheat or flour protein with increase in loaf volume, but wet crude gluten and gluten protein do seem to increase in quantity in proceeding from top to bottom in this table, and the same trend is somewhat noticeable in the results showing the soluble protein. The variability in protein content among the wheats and flours is not very large, and certainly cannot account for the relatively large differences in loaf volume. This situation is somewhat different from previous researches conducted by the author on hard red spring wheat, when large variability existed in crude flour protein, these variations being closely associated with variability in loaf volume. The strength of this association appeared to be sufficient to justify the use of total flour protein as a means of forecasting flour strength from a knowledge of wheat or flour protein. The wet crude gluten values are somewhat more variable than the crude protein results. Large differences in the physical quality of the glutens were evident during the washing procedure, and it was quite easy to differentiate between the flours yielding satisfactory and poor loaves by the "feel" of the washed crude gluten. The glutens in the present study were not difficult to wash in contrast to a few of the durum wheat flours previously examined. The quantity of protein extracted by sodium salicylate varies to some extent among the various flours. The ash content of the flours ranges from 0.27 to 0.56% and this rather large difference is probably due in a large degree to the difficulty of milling such extremely hard and vitreous wheat as was produced in the 1936 crop season by exposure to abnormal growing conditions of drought and heat.

In Table II are shown the results obtained by fractionating the dispersed gluten protein through the addition of 3, 8 and 10 c.c. of concentrated $MgSO_4$ solution, the fractions thus obtained being designated as fractions 1, 2 and 3, respectively, for the sake of brevity. The total protein removed from solution by the combined additions of $MgSO_4$ is also shown, and represents the sum of the three fractions. It is evident that a considerable quantity of gluten protein remains in solution, and this quantity varies from sample to sample. A regular increase in protein thrown down as fraction 1 is noted with increasing loaf volume, but the other fractions do not show the same trend. This is in line with the work published by Harris (1937) dealing with similar studies on durum wheat flours. Great variability was found in the results obtained for the second and third fractions, and it did not seem possible to obtain the same precision in these cases as was achieved

TABLE II

PROTEIN (N × 5.7) FRACTIONS[1] PRECIPITATED FROM SODIUM SALICYLATE SOLUTION BY SUCCESSIVE ADDITIONS OF $MgSO_4$

Sample number	Fraction 1		Fraction 2		Fraction 3		Total protein removed from solution	
		Percent of soluble		Percent of soluble		Percent of soluble		Percent of soluble
	Mg.		*Mg.*		*Mg.*		*Mg.*	
37–1–74	66.7	15.3	233.2	53.5	19.4	4.4	319.3	73.2
81	89.5	20.9	207.4	48.5	44.4	10.4	341.3	79.8
133	78.7	18.9	230.3	55.3	57.6	13.7	366.6	88.0
80	75.2	16.9	226.2	50.9	46.8	10.5	348.2	78.3
137	86.6	19.7	253.1	57.7	51.3	11.7	391.0	89.1
68	68.4	14.8	207.0	44.8	79.6	17.2	355.0	76.8
71	120.3	23.1	260.0	49.8	64.4	12.3	444.7	85.2
77	112.3	20.6	238.8	43.9	67.2	12.3	418.3	76.8
79	120.3	23.6	220.0	43.1	67.2	13.2	407.5	79.9
78	123.1	24.0	225.8	44.0	61.0	11.9	409.9	79.9
76	130.0	24.1	247.4	45.9	64.4	11.9	441.8	82.0
72	80.9	18.7	297.6	68.7	68.4	15.7	446.9	103.1
83	126.0	26.1	233.6	48.5	48.4	10.0	408.0	84.7
73	92.9	19.5	306.6	64.4	10.2	2.1	409.7	86.0
75	107.7	22.5	174.4	36.4	13.2	2.7	295.3	61.7
62	92.3	18.4	238.8	47.6	81.0	16.1	412.1	82.1
135	108.9	23.6	236.5	51.2	55.9	12.1	401.3	86.9
64	117.4	21.3	252.6	45.9	70.2	12.8	440.2	80.0
69	132.8	20.6	311.8	48.4	74.1	11.5	518.7	80.5
65	109.4	21.4	240.0	47.0	72.4	14.2	421.8	82.6
70	130.5	24.1	245.6	45.4	67.2	12.4	443.3	81.9
115	139.6	28.6	241.1	49.4	59.3	12.2	440.0	90.2
95	137.4	29.0	232.0	49.0	51.8	10.9	421.2	88.9
37–2–9	132.8	25.6	265.6	51.2	55.8	10.7	454.2	87.5
37–1–109	137.4	28.2	245.1	50.2	61.6	12.6	444.1	91.1
37–2–3	107.2	20.9	244.0	47.6	71.8	14.0	423.0	82.5
37–1–101	126.0	26.3	242.8	50.7	54.2	11.3	423.0	88.3
37–2–2	114.0	23.0	244.6	49.3	72.4	14.6	431.0	86.9
11	155.0	29.7	227.4	43.6	69.6	13.3	452.0	86.6
10	161.9	31.9	207.0	40.8	64.4	12.7	433.3	85.4

[1] Fractions 1, 2 and 3 refer to quantities of protein precipitated by 3, 8 and 10 c.c., respectively, of $MgSO_4$ solution.

in the instance of the determination of the initial fraction. The maximum quantity of protein was removed by 8 c.c. of $MgSO_4$, and decreased sharply upon the addition of a further 10 c.c. of the reagent. No effort was made to calculate the total protein removed by each successive addition until the total 21 c.c. had been run in.

The correlation coefficients calculated between the various variables are shown in Table III, with corresponding tests of significance. Protein fraction 1 is significantly and positively correlated with loaf volume, this also being the case with percent of wet crude gluten and

TABLE III

CORRELATION COEFFICIENTS COMPUTED FROM THE FRACTIONATION AND BAKING DATA

Variables correlated		r_{xy}	P [1]
X	Y		
Loaf volume	Crude flour protein	+.0818	>.5534
Loaf volume	Wet crude gluten	+.6482	<.0002
Loaf volume	Gluten protein in flour %	+.5532	.0016
Loaf volume	Protein fraction 1 (mg.)	+.7769	<.0001
Loaf volume	Protein fraction 1 (% of soluble)	+.7277	<.0001
Loaf volume	Protein fraction 2 (mg.)	+.1098	>.5534
Loaf volume	Protein fraction 2 (% of soluble)	−.2544	.1786
Loaf volume	Protein fraction 3 (mg.)	+.3815	.0380
Loaf volume	Protein fraction 3 (% of soluble)	+.2278	.2258
Loaf volume	Total protein removed from solution (mg.)	+.6253	.0002
Loaf volume	Total protein in solution (mg.)	+.4616	.0104
Protein fraction 1 (mg.)	Wet crude gluten	+.2895	.1208
Crude flour protein	Wet crude gluten	+.3540	.0552
Crude flour protein	Gluten protein in flour %	+.6075	.0004
Total protein in solution (mg.)	Total protein removed from solution	+.7095	<.0002
	yz	$r_{x(yz)}$	
Loaf volume	Wet crude gluten and protein fraction 1 (mg.)	+.8939	

[1] = probability of the observed correlation coefficient arising from uncorrelated mater al through errors of random sampling.

gluten protein of the flour, while crude flour protein is quite unrelated to baking strength. The remaining fractions do not have a relationship of any great importance to baking strength. The total quantity of protein removed by $MgSO_4$ is, however, correlated with this variable, as is soluble protein to a lesser degree. The coefficient between crude flour protein and wet crude gluten approaches the horizon of significance, while the first variable and percent of gluten protein in the flour are more highly related. A significant positive correlation was found to exist between the quantity of protein dispersed by sodium salicylate solution and the total protein removed by the three portions of $MgSO_4$ solution added. The dispersed protein in milligrams is also positively related to loaf volume, and this finding agrees with the work reported by Sinclair and McCalla (1937) who postulated a decrease in gluten solubility in sodium salicylate in relation to baking strength when dealing with deteriorated flours.

A decided gain of information was obtained relative to loaf volume by fractionating the gluten protein, as is proved by the test of significance of the multiple correlation which measures the combined influence of wet crude gluten content and fraction 1 upon loaf volume. These latter data are shown in Table IV.

Comparative data between hard red spring and durum wheats are shown in Table V, the values for the durum varieties being taken from the study of durum wheat protein fractionation previously cited. The crude protein content of wheat and flour is higher for the durums, justifying the conclusion held by some that durum wheat tends to

TABLE IV

TESTS OF SIGNIFICANCE OF ADDITIONAL INFORMATION GAINED BY CALCULATING MULTIPLE CORRELATION COEFFICIENTS

Sum of squares		*D F*	Variance	*F*	5% Pt.
$1 - (.6482)^2$	.579837	28			
$1 - (.8939)^2$	.200943	27	.007442		
Difference	.378894	1	.378894	50.91	4.21
$1 - (.7769)^2$	.396426	28			
$1 - (.8939)^2$	.200943	27	.007442		
Difference	.195483	1	.195483	26.27	4.21

TABLE V

COMPARISON BETWEEN DURUM AND HARD RED SPRING WHEATS IN REGARD TO PROTEIN, ASH, AND GLUTEN FRACTION VALUES

	Crude protein (N ×5.7)		Ash	Wet crude gluten	Gluten protein (N ×5.7) in flour	Protein fractions[1]			Total protein removed	Soluble protein (N ×5.7)
	Wheat	Flour				1	2	3		
	%	%	%	%	%	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*
Hard red spring	16.8	14.7	0.43	45.6	13.0	112.7	241.2	59.8	412.1	493.8
Durum	17.4	16.5	0.85	50.8	13.3	47.0	199.4	106.6	353.0	445.7

[1] Quantity of protein precipitated by 3, 8 and 10 c.c. of $MgSO_4$, respectively, from 100 c.c. of dispersion.

run higher in protein than common wheat varieties. The ash content is also much higher. The crude gluten content is higher in the durums but percent of gluten protein in the two classes of flour is almost identical. A comparison of the fractionation data for the two classes of wheat shows that the durum had a much smaller quantity removed as fraction 1, this difference decreasing with fraction 2, and being reversed in the instance of the third fraction. This result is in accord with the evidence presented by Harris and Bailey (1937) who found larger proportions of "gliadin" present in the non-*vulgare* wheat varieties. Presumably smaller protein particles exist in the final fractions removed by the procedure employed by Harris and Bailey and the method used in the present instance. Both total protein removed and protein solubility are lower in the case of the durum wheats as compared with hard red spring varieties.

Comparative data assembled from analyses of the six flours experimentally milled from a sample of hard red spring wheat of 16.6% protein to represent as closely as possible commercial "millstream" divisions are shown in Table VI. The differences in ash content of these flours point to the fact that materials from various portions of the wheat berry must have been incorporated in these classifications.

The flour from fifth break and tailings was extremely high in ash, and produced a loaf with the lowest color and loaf volume. This sample had also the highest crude protein and gluten content. Color was best in the instance of sample 2, which had good texture and the lowest ash. A rapid rise in ash occurred as the tail end of the process was approached, associated with a poorer crumb color. The crude protein increases in the same order, as is the case with wet crude gluten. Diastatic activity (Blish-Sandstedt)is at a minimum in sample 1, with a maximum being reached with sample 5, showing the inclusion of poor quality material with high enzymatic content in this flour. This sample also produced the poorest loaf. Moisture, of course, decreased with length of extraction and the associated longer time of exposure to the drying effect of the air.

In Table VII are given the fractionation and gluten protein values for the six flours. The protein removed by 3 c.c. of $MgSO_4$ solution was the sole fraction isolated, as it has previously been shown that

TABLE VI

COMPARATIVE DATA ON EXPERIMENTALLY MILLED FLOURS PRODUCED FROM ONE WHEAT SAMPLE AND CLASSIFIED TO REPRESENT DIFFERENT "MILLSTREAM" SEPARATIONS (13.5% MOISTURE BASIS)

Separation	Laboratory number	Crude protein (N × 5.7)	Moisture	Ash	Diastatic activity—maltose produced by 10 g. flour	Wet crude gluten	Loaf volume	Color	Texture
		%	%	%	*Mg.*	%	*C.c.*	*Score*	*Score*
1st sizings and 1st middlings	37–5–1	15.0	13.7	0.47	98.3	47.4	608	94	95
2nd, 3rd and 4th middlings	2	14.4	13.3	0.46	125.0	45.4	583	95	95
5th middlings, 1st tailings	3	15.8	12.0	0.68	170.0	50.2	612	94	95
2nd and 3rd break	4	17.8	13.7	0.72	130.0	56.5	670	93	92
5th break, tailings	5	20.3	11.3	1.52	229.0	66.9	555	86	93
1st and 4th break	6	19.2	13.3	0.82	126.7	66.1	624	92	94

TABLE VII

SOLUBLE PROTEIN, PROTEIN FRACTIONS AND GLUTEN PROTEIN IN FLOURS MILLED TO REPRESENT "MILLSTREAM" SEPARATION

Sample number	Soluble protein mg. for 100 c.c.	Protein fraction		Protein (N×5.7) in gluten	Gluten protein (N×5.7) in flour
			Percent of soluble		
		Mg.		%	%
37–5–1	501	146.6	29.3	29.3	13.9
2	507	161.8	31.9	28.2	12.8
3	501	155.8	31.1	27.9	14.0
4	508	165.2	32.5	28.2	15.9
5	456	119.6	26.2	24.4	16.3
6	499	154.2	30.9	26.3	17.4

further fractionation does not yield increased information in regard to flour strength. Considerable variation in protein solubility is evident, the best flours appearing to be the more easily dispersed in sodium salicylate, and the sample producing the largest loaf, sample 4, has the largest quantity of protein thrown down. On the other hand, the flour yielding the smallest loaf has the lowest quantity of protein removed by 3 c.c. of $MgSO_4$ solution. It would appear that the protein fractions vary among flours milled from the same wheat sample in much the same manner as among flours produced from various wheats. A similar conclusion was reached in regard to peptization by Harris (1937) in examining a series of commercially milled millstream flours produced from the same wheat blend.

The final picture of the gluten protein situation appears to the author at this stage as follows: wheat flour gluten may be reversibly fractionated into a large number of fractions which vary in appearance from a substance rather closely resembling the original gluten and which is easily separated into a hard, compact mass by centrifuging, to other constituents of a less viscous consistency which may be pulled into thin threadlike strands of great tenacity. It became more difficult to separate the later fractions as the protein adhered more tenaciously to any substance with which it came in contact. The initial protein removed appears to be rather highly related to flour strength in various types of wheat, and further work is programmed in these laboratories along this line of investigation. It is difficult to say whether the gluten proteins consist of one individual complex which may be split under appropriate treatment into a large number of fractions each differing in slight degree from its neighbor, or whether the fractions are thrown down as mixtures, each separation containing different proportions of the original constituent complexes. The author leans somewhat toward the latter viewpoint and is inclined to believe with Blish and Sandstedt (1933) that there are probably three distinct gluten proteins, or rather gluten complexes which differ among themselves in physical and chemical properties.

Summary and Conclusions

A series of 30 samples of flour experimentally milled to a 70% extraction from hard red spring wheat was analyzed for crude flour protein, ash, moisture, and wet crude gluten content. The gluten nitrogen was determined and the protein in the gluten computed. The flours were also baked by the standard A. A. C. C. method using 5% of sucrose. The glutens washed from the flours were then dispersed in 10% sodium salicylate solution, and fractionated by the successive additions of 3, 8 and 10 c.c. of $MgSO_4$ solution.

Fraction 1 was highly correlated with loaf volume, while the remaining two fractions had little relationship with flour strength. Crude flour protein was not related to loaf volume, while, on the other hand, percent of wet crude gluten and gluten protein were positively correlated with this variable. The same relationship was also apparent between total protein removed by $MgSO_4$ and loaf volume. A smaller correlation coefficient was found between loaf volume and the total amount of protein dispersed by sodium salicylate solution. The proportion of total protein removed from the salicylate dispersion appeared to vary with the solubility of the gluten in the dispersion medium.

A gain of information in respect to loaf volume was secured through determining the proportion of protein isolated by the initial volume of $MgSO_4$ added, over that furnished by a knowledge of wet crude gluten content.

A comparison between hard red spring and durum wheats showed the crude protein content of wheat and flour to be higher for the durums. The durum as a class had a smaller quantity of protein in the first fraction. This difference was less for fraction 2 and in the instance of the final fraction the hard red spring varieties had less protein precipitated. Both total protein removed and protein solubility are lower in the case of the durum wheat as compared with hard red spring varieties.

Further work of a similar nature was undertaken on six flours milled from a sample of hard red spring wheat to approach as closely as feasible to commercial millstreams. Only the first protein fraction was isolated in this series. This fraction appeared to vary among the different flours, the sample baking into the best loaf having the largest quantity of protein precipitated, while the poorest quality flour had the smallest proportion of protein thrown down. The fractions from the other flour samples varied between the two limit values. The six flours described appeared to approach quite closely to corresponding commercial samples in their chemical characteristics.

Acknowledgment

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A SENSITIVE METHOD FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITY

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Introduction

According to Bergmann (1936) proteases may be classified into these which split off terminal acids with a free carboxyl group, terminal acids with a free amino group, and CO−NH or true peptide linkages within the chain. Accepting the recently developed theories of chain to chain coordination (through −N→HO− and −O→HN-semi-polar bonds) according to which the essential colloidal properties of micellar aggregates of long chain polymers are manifestations of the increased particle size caused by the action of these secondary valence forces, it can be seen that the scission of a peptide link within a chain would have much more effect in changing these properties than the elimination of a terminal unit. Each, of course, would produce the same increment in free amino groups which is so frequently used to determine protein degradation by such methods as that of Van Slyke, formol titration, etc.

Since in the study of gluten we are most interested in changes in colloidal properties, and in malting and mashing in the production of proteoses and peptones, *i.e.*, in the scission of deeply intra-chain linkages, a method designed to emphasize such degradation and to minimize the effects of the splitting of terminal units is desirable. Colloidal properties being particularly sensitive to scissions within the chain, a physico-chemical method is indicated. Gelatin, a widely used substrate, possesses particular advantages for use in such a method.

Substrate

In the natural state collagen undoubtedly exists in an exceedingly complex structure; its mechanical strength indicates the unusual character of the coordination tendencies. The partially degraded derived protein gelatin possesses these properties to a lesser, but still appreciable, degree. It is by no means a uniform material (the molecular weight of a single sample may vary from 6,000 to over 200,000

according to Krishnamurti and Svedberg (1930)) and each lot will possess different properties depending upon the method of manufacture. Moreover, it is extremely sensitive to electrolytes, temperature, concentration, etc. (Kraemer, 1926). This very sensitivity, however, becomes a valuable characteristic in the study of small concentrations of proteolytic enzymes, provided other factors can be adequately controlled. The unique property of gelatin by which inter-chain coordination is magnified in the formation of gels makes detection of small changes a feasible proposition.

The collagen proteins in their many derived forms have long been used qualitatively and semi-quantitatively for the determination of proteases. Numerous investigators have used the gelation property in particular. The difficulty of exactly controlling conditions and the lack of knowledge regarding the effect of variations in the environment have somewhat limited the usefulness of such procedures. In the method to be described great pains have been taken to control temperature, gelatin and electrolyte concentrations and pretreatment of the substrate, which have resulted in a procedure that can be reproduced from day to day with confidence.

Gelation Rate Characteristics

It has been found that the rate of gelation, as indicated by increase with time of the viscosity, is susceptible to measurement and is directly related to the changes produced by proteolytic enzymes. Under the conditions of the experiment this function, *i.e.*, viscosity as a function of time of gelation, is given by

$$\frac{d\eta}{\eta\, dt} = a$$

or

$$log\ \eta = at + b$$

and further, changes in this rate are, over a limited range, directly proportional to the concentration of certain proteolytic enzymes:

$$a = kc + a_0.$$

Here η is the apparent viscosity at time t, c is the concentration of enzyme and k a constant. The calculations are greatly simplified by the curious fact that the logarithmic gelation rate curves intersect at a common point not the origin after extrapolation. (Figure 1.) The reason for this unique behavior has not been fully elucidated.

The relationship between ionic strength and gelation rate has not yet been fully investigated, but it appears that at total electrolyte concentrations greater than 0.05 M the rate of change, while still

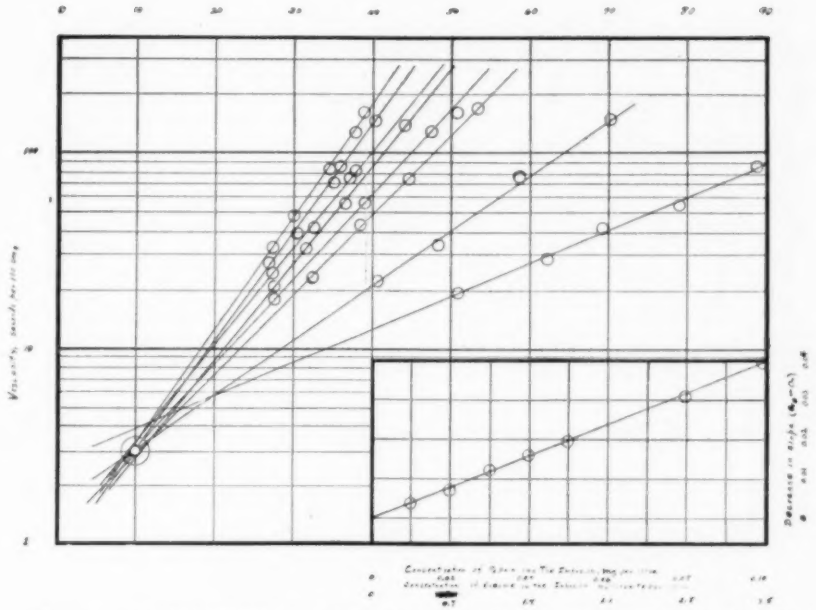

Figure 1. A typical calibration. In order of decreasing slope the curves represent the logarithmic gelation rates of a 0.75% gelatin solution after digestion for 20 hours with concentrations of papain equivalent to 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.08 and 0.10 mg. per 100 c.c. in the infusion. Extrapolation of each curve to the point of intersection gives the value of the pole, taken here as $\eta = 3$ seconds per 100 mm., $t = 10$ minutes. By the Gore (1929) method the activity of this sample of papain was found to be 35.0 units per gram. From these values the data represented in the lower right hand corner are obtained. The slope of this curve is 89.6 milliunits of activity per 100 c.c. of infusion per unit change in slope, which then becomes the calibration figure to be used for this procedure.

appreciable, is small. Hence a citrate-phosphate buffer at 0.06 M final concentration has been used. For the particular pork-skin gelatin adopted the pH-gelation rate curve has a maximum at pH 5.0 to 5.5 (Figure 2). The measurements are made within the range,

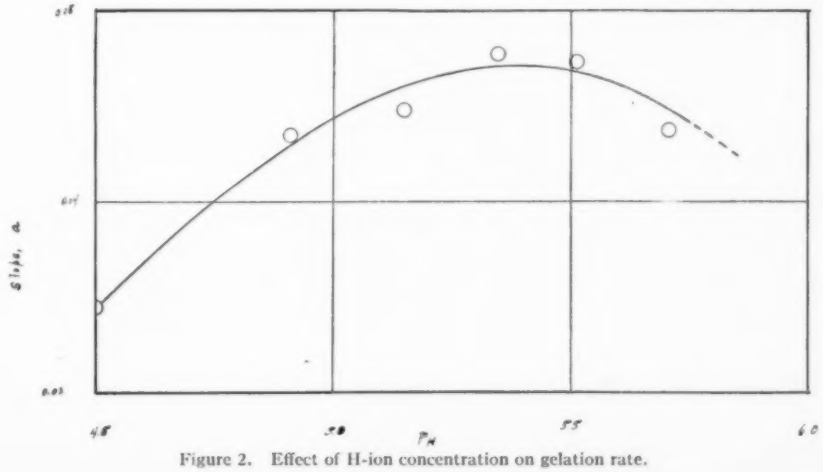

Figure 2. Effect of H-ion concentration on gelation rate.

which is the normal range of dough fermentation and not far removed from that existing in mashing practice.

The structure of a gelatin gel is commonly thought to be a complex brush-heap aggregation of micellar units. The growth of this structure is so markedly affected by temperature that slight irregularities are sufficient to produce a nonuniform gel in which inhomogeneities may be readily discerned. The structure is also very sensitive to mechanical shearing; once stirred or sheared the apparent viscosity of the gel decreases markedly. Fortunately, however, under the conditions to be described gelation proper is preceded by a stage of a-micronic aggregation, during which the solution exhibits true viscosity and is not susceptible to changes induced by shearing. If cooling of the solution during this stage is accompanied by gentle stirring, a sample without local inhomogeneities of temperature is obtained which will gel uniformly.

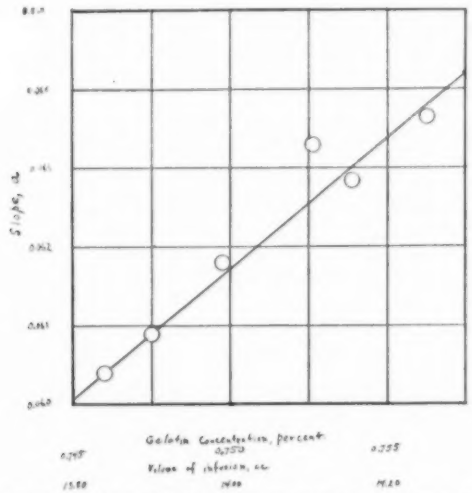

Figure 3. Effect of gelatin concentration on gelation rate.

It appears that gelatin, in common with a number of other such colloids, exhibits a temperature—degree of aggregation equilibrium. For example a gelatin sol prepared at a temperature below 40° C. when allowed to stand at 40° will gradually decrease in gelation rate (measured at 25° C.), while if prepared at 50° or higher it will gradually increase in gelation rate. Hence after preparation a certain interval must elapse before the use of the substrate in order that equilibrium may be approached.

Maintenance of a constant concentration of gelatin is of vital importance. The change in gelation rate produced by a small change in concentration over a small range is illustrated in Figure 3. It will

be seen that while the change is considerable it can be reduced to negligible proportions by using careful analytical precautions and keeping pipets well cleaned.

General Considerations

Since the reaction cannot be stopped without seriously imparing the gelation characteristics, it is necessary that the digestion period and testing time be so proportioned that the influence of the latter in the conversion measured is negligible. Partly to conform to this requirement and partly to increase the sensitivity, a digestion time of 20 hours at 30° C. has been adopted. As the testing is done at 15° C., where the reaction velocity is at least halved, and rarely requires more than 60 minutes, the amount of conversion occurring during testing must be less than 2.5% at most and is usually less than 1% of the total.

In view of the nonuniformity of gelatin and the lack of standardization of viscometers, thermostats and similar items of equipment, it is obvious that the method can hardly be put on an absolute basis. Consequently each unit of gelatin, each modification of procedure, must be calibrated in terms of a sample of known activity or of another method. We have used the method of Gore (1929) which, while lacking in sensitivity, depends on the changes induced in the mutarotation of gelatin, and is less dependent on the particular sample of gelatin used. Other methods applicable only to relatively active enzyme concentrates may also be used to calibrate the sensitive procedure.

Experimental

Apparatus

Viscometer:—An eccentric falling ball type. Types in which any shearing of the gel may occur before test are unsuitable. One with interchangeable viscosity tubes is to be preferred, as the capacity is severely limited if only one tube is available. This viscometer should be completely submerged in the low temperature thermostat. A set of twenty matched viscosity tubes is a recommended accessory. Wall thickness should also be reasonably well matched.[1]

Thermostat, high temperature:—A high temperature thermostat capable of regulating to within 0.05° C. This is set at 30° and is used for the 20-hour digestion.

Thermostat, low temperature:—A low temperature thermostat capable of regulating to within 0.001° C. and containing a device for

[1] Jena KPG precision bore tubing, 16.00 ± 0.01 mm. i.d., distributed by the Fish-Schurman Corporation, New York, has been used. A Hoppler viscometer ball 15.45 mm. in diameter appears to be suitable for these conditions.

rotating the viscosity tubes within the bath. This is normally set at 15° C. The construction of so-called ultra thermostats is a rather difficult and specialized task but it might be stated that cooling, heating, stirring and thermo-regulator lag must be properly balanced. Even then hunting is likely to occur if a heavy load of warm tubes is placed on the system.

Special pipets:—Calibrated to about one cubic centimeter less than half the capacity of the viscometer tubes. For KPG tubes 16.00 mm. i.d. by 6.63 inches long, uniformly closed by tightly fitting rubber stoppers, a pipet delivering 14 c.c. is suitable.

Clock, stopwatch:—Conventional analytical equipment.

Reagents

Distilled water:—Apply a vacuum of 5–8 mm. Hg to a sample of distilled water free from metallic contamination for several hours, not allowing the temperature of the water to drop much below room temperature. This removes most of the dissolved gases.

Buffer:—Dissolve 153.8 g. citric acid (0.8 mol) and 573.2 g. $Na_2HPO_4 \cdot 12H_2O$ (1.6 mol) in water, then make to a final weight of 4,370 g. with water. The pH should be between 5.0 and 5.5. After mixing separate into small samples of 200 c.c. each and sterilize in an Arnold.

Substrate:—Weight accurately into a beaker of convenient size the amount of gelatin which has been found by preliminary tests (see below) to give a convenient gelation rate (*e.g.*, 3.75 g.—see Figure 3). Add not more than 10 times this weight of cold water (25 c.c.), stir, and after a thick paste containing no dry lumps of gelatin has been obtained transfer the whole to a double boiler (nested beakers, 50 c.c. and 100 c.c., respectively, are suitable). Stir carefully, avoiding foam formation, until a homogeneous solution is obtained (about 2 to 3 minutes are sufficient if not more than 5 g. of gelatin are being prepared). Pour into a flask containing 10 to 20 c.c. of hot water and rinse three times with hot water, using precautions customary to quantitative analysis. Add the required amount of buffer solution such that the concentration in the final reaction mixture will be 0.06–0.08 M (50 c.c of buffer solution to 250 c.c. of substrate solution have been found satisfactory). Now make up with water to a final weight corresponding to that previously found to give a suitable gelation rate. It should be noted that this concentration is twice that existing in the final reaction mixture. Place in the bath at 30° C. to age for the required time (10 hours has been found satisfactory). This solution should now never be cooled below 30° C. until the final test for gelation

rate, else the equilibrium will be disturbed. It should be preserved under toluene.

Enzyme infusions:—Prepare enzyme infusions such that the final infusion contains from 1 to 3 milliunits per 100 c.c. For finely powdered materials of low activity which disperse easily, such as flours, drop the sample as a thin stream into a flask of water while rotating the flask. Allow to macerate for one hour, shaking at intervals. Then centrifuge and use the supernatant liquid for the determination, or a suitable aliquot thereof. For syrups disperse in the usual manner. For highly active powdered or coarsely ground concentrates such as papain weigh the sample (usually not less than 50 mg.) into a glass or agate mortar, triturate with a few drops of water, and wash quantitatively into a volumetric flask. Suitable concentrations are given in Table I.

TABLE I

RECOMMENDED CONCENTRATIONS

Material	Effective concentration in the infusion, per 100 c.c.
Wheat flour	3–5 g.
Dry malt extracts	3–5 g.
Liquid malt extracts	1–3 g.
Malt flours	0.01–0.2 g.
Animal digestive concentrates	1–50 mg.
Papain	0.05–2 mg.

PROCEDURE

The procedure cannot be described in absolute terms. For the substrate a large sample of gelatin should be obtained. A commercial food gelatin prepared from pork skin, moisture 10.0%, pH 4.5, isoelectric point 5.35 pH, has been used. It is first necessary to determine the concentration of gelatin required to give a satisfactory gelation rate. Following the procedure given for the preparation of the substrate various concentrations are investigated. It may be advisable to study both the pre-gel and gel stages, and a concentration selected which will give a common (Briggsian) logarithmic gelation rate of about 0.06 per minute. This choice is governed by the relative errors of determination of the time of fall of the ball in the viscometer and the precision of reading the time of gelation. Ordinarily the former is recorded to the nearest 0.2 second; the latter to the nearest 0.1 minute. Hence if the time of fall of the ball is regulated to be about 60 seconds (not over 200 centipoises, absolute measure) at 30 minutes the degree of precision of the two readings is of the same order of magnitude.

The general procedure in this determination (of suitable gelatin concentration) is as follows: An arbitrary concentration of say 0.75% gelatin is chosen for trial. Then 3.75 g. of gelatin are weighed out and made up finally with water to 250 g. including buffer as described above. After 10 hours at 30° C., 14 c.c. portions are transferred to a series of viscometer tubes, 14 c.c. of distilled water added, the contents mixed, and after 20 hours at 30° C. the testing of gelation rate is begun. The low temperature thermostat is adjusted to 15° ± 0.001° C. At regular intervals, say 2.5 minutes, the tubes are transferred one at a time from the 30° bath to the rotating device in the 15° bath, the time of transfer being recorded to the nearest 0.1 minute. After 10 minutes of rotation each tube is successively transferred to a stationary vertical position. Beginning at say 15 minutes after introduction into the 15° bath, the tubes are transferred successively to the viscometer and the viscosity determined at say 2- to 5-minute intervals, the time at which the viscosity test is begun being recorded to the nearest tenth minute, the time of fall being recorded to the nearest fifth second. The gelation time is then corrected to the mid-point of the viscosity determination. The data are then plotted on semi-log paper. It will be found that after a certain time, perhaps 25 minutes, the function becomes linear in log η. In actual use measurements should be confined to this portion of the curve, the upper limit being that at which the viscosity determination becomes uncertain due to the great length of time required for the fall of the ball. The experiment is repeated with varying concentrations until a convenient value such as $\frac{d \log_{10} \eta}{dt} = 0.06$ (min.$^{-1}$) is obtained.

Having decided upon the concentration of substrate to use, the next step is to determine the pole, *i.e.*, the point of intersection of various gelation rate curves. (Figure 1.) A batch of substrate is prepared, aged, and 14 c.c. portions (or other suitable volume) pipetted into the viscometer tubes. Now 14 c.c. portions of enzyme infusions containing 0.1 milliunit increments of enzyme are added, the tubes stoppered, the contents thoroughly mixed, and digestion allowed to proceed for 20 hours. Enough replicates (at least two) of each concentration of enzyme should be included to determine each log $\eta - t$ function. The solutions are then tested as described previously. It is convenient to determine the pole graphically by plotting η vs. t on semi-log paper. Since the extrapolation involved is considerable, an average value must be taken for the pole. This value is then used as one point on the curve in the calculation of subsequent results. For the conditions we have used the coordinates of the pole are $\eta = 3$ sec./100 mm., $t = 10$ minutes.

Calibration

Because the method is so highly empirical, each procedure must be calibrated. After the routine is fairly well established a standard sample of known activity may be run. The slope,

$$a = \frac{d\eta}{\eta\, dt} = \log\left(\frac{\eta}{\eta_p}\right)(1/(t - t_p)),$$

where η is the viscosity of the sample, η_p the viscosity coordinate of the pole, t is the corrected time of gelation and t_p the time coordinate of the pole, is calculated for the blank and for the active runs. The difference $(a_o - a)$ is plotted against the known concentration of enzyme units in the infusions. The slope of this curve gives the calibration of the procedure in terms of units of activity per unit change in the slope, $d\eta/\eta\, dt$. For the procedure here described this

TABLE II

Typical Set of Data. Digestion Time, 20 Hours at 30°. Pole: $\eta = 3$, $t = 10$

No.	Sample	Concentration in infusion g./100 c.c.	Gelation time, minutes				Viscosity, η, seconds		
			Start	Test	Time	Time corr.	Tube length, mm.	"Fallzeit" obs. per 100 mm.	
1	Blank	0	810.0	838.9	28.9	29.2	100	39.2	39.2
2	Flour	6	812.5	894.7	82.2	82.6	50	48.2	96.4
3	"	4	817.6	877.8	60.2	60.6	50	47.2	94.4
4	"	3	820.0	864.5	44.5	44.9	100	47.8	47.8
5	"	2	827.5	870.4	42.9	43.2	50	41.2	82.4
6	"	1	830.0	868.7	38.7	39.1	50	46.4	92.8
7	"	0.05	832.6	866.8	34.2	34.7	100	62.2	62.2

Calculations on the Preceding Data

No.	log $(\eta/3)$	$\frac{\log(\eta/3)}{t - 10} = s$[2]	$s_0 - s$	$90(s_0 - s)$ = milliunits per 100 c.c. of infusion	Proteolytic activity, milliunits per gram
1	1.116	$0.0582 - s_0$	—	—	— (blank)
2	1.507	0.0208	0.0374	3.36	0.57 [1]
3	1.498	0.0297	0.0285	2.56	0.64
4	1.202	0.0346	0.0236	2.15	0.71
5	1.439	0.0433	0.0149	1.34	0.67
6	1.491	0.0513	0.0069	0.62	0.62
7	1.317	0.0534	0.0048	0.43	0.86 [1]
				Average	0.66

[1] Omitted from the average.
[2] s in the table = a in the text.

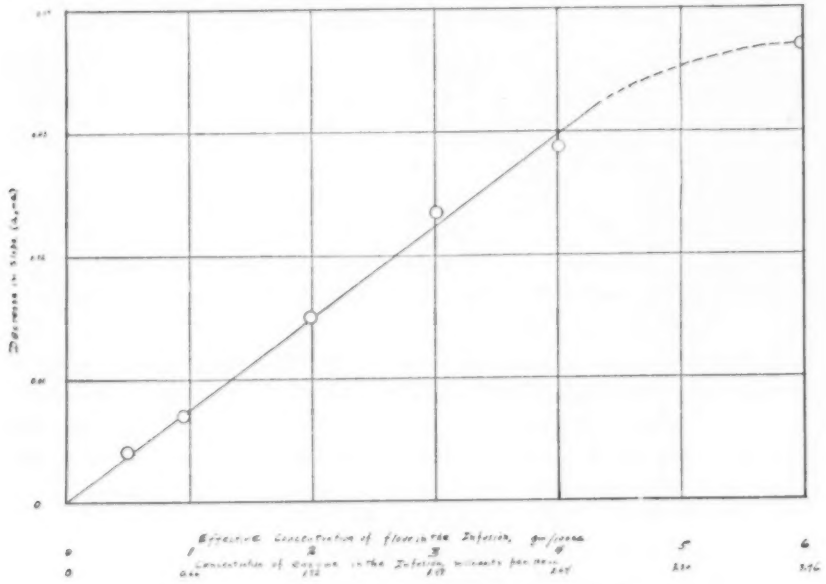

Figure 4. Allowable enzyme concentration range: Kansas flour.

TABLE III

SOME TYPICAL VALUES

Material used	Concentration g./100 c.c.	Activity, milliunits per gram
Flours		
N. W. short patent	3	0.43
N. W. high gluten	3	0.99
Kansas short patent	3	0.50
Texas patent	3	0.66
N. W. patent, unbleached	3	0.62
Same, bleached	3	0.41
Kansas, unbleached	3	0.61
Same, bleached	3	0.42
Soya flour	0.5	3.22
Barley malts		
6-day green malt	0.5	6.10
Pale Minn. Brewers'	0.5	5.03
2nd grade Vacuum Distillers	0.5	4.76
Malt extract, 1	1	2.34
Malt extract, 2	3	0.73
Malt extract, 3	3	0.20
Bakers' yeast	2	0
Same, treated with $CHCl_3$ for 24 hours	0.2	12
Bacterial amylase	0.2	27
Pancreatic amylase	10^{-3}	1,400
Papain 1	10^{-4}	12,300
Papain 2	10^{-4}	45,700

value is 90 milliunits per 100 c.c. per unit change in slope, based on the Gore method (*loc. cit.*).

A typical set of data and calculations is given in Table II. This also illustrates the range within which enzyme concentrations must be kept. (Figure 4.) The values obtained for some common materials are given in Table III.

Summary

Dilute gelatin sols in equilibrium at 30° C., when cooled to 15° C., increase in viscosity, exhibiting true viscous flow before the onset of gelation proper. Gelation then proceeds according to the equation $\frac{d\eta}{\eta\, dt} = a$, where η is apparent viscosity at time t and a is a constant characteristic of the conditions. When digested with proteolytically active infusions a decreases, and over a limited range the decrease is directly proportional to enzyme concentration. This forms the basis of the sensitive method.

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GERM CONTENT OF AMERICAN WHEATS[1]

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Widely varying reports appear in the earlier literature respecting the percentage of germ or embryo in wheat kernels. Girard (1897), working with four French wheats, reported 1.16 to 1.50%; Fleurent (1896) found 1.50% in Algerian, 2.00% in Russian, and 2.05% in Canadian wheat; Osborne and Mendel (1919) reported 1.5%, presumably in American wheat; Percival (1921) recorded 2.8 to 3.5%; Grischenko (1935), of the Cereal Research Institute in Rostow/Don (U. S. S. R.), found 2.63 and 2.83% in two samples of the wheat variety Ukranka, and 2.56, 3.07, 2.81 and 3.25% in four samples of the variety Melanopus 069. Thus the percentages reported by Percival and Grischenko are considerably higher than the earlier findings. On the other hand Ingersoll and Bessey (1893) stated that the germ constituted 6% of the kernel, although the method of determination employed by them was not described and one is led to assume that they merely estimated the proportion from the apparent volume as seen by the microscope. Dedrick (1924) recorded the same estimate, possibly taken from Ingersoll and Bessey's statement.

It appeared desirable, in the light of these varying data, to ascertain the average of, and relative variations in the germ content of certain typical American wheats. To this end, several samples were collected by the wheat purchasing department of General Mills, Inc., which were regarded as representative of various types of hard spring, hard winter, and durum wheats. In addition, three samples of certain soft wheats were also secured.

There are no standard methods or techniques for the dissection of cereal kernels, and some preliminary experimentation was necessary to develop an acceptable procedure. After trying several methods the following was adopted for the purpose of this study: About 200 kernels, freed from broken and damaged grains, weed seeds and other foreign matter, were submerged for about five minutes in boiling water. They were then removed from the hot water, and allowed to stand for an additional 10 minutes. This treatment served to facilitate the subse-

[1] Paper No. 11, Journal Series, General Mills Research Laboratories.
[2] With the technical assistance of Catherine Becker.

quent detachment of the periderm over the surface of the germ and the later removal of the germ from the cavity in which it rests.

Two samples of 50 kernels each were then counted out at random. Using an eye surgeon's iris knife (see Figure 1), the periderm directly over the germ of each kernel was split, and peeled back. The germ was carefully cut loose at the edges. Gentle pressure on the starchy endosperm at the sides of the germ caused the embryo to slide out intact. The 50 germs thus separated from each sample were placed in

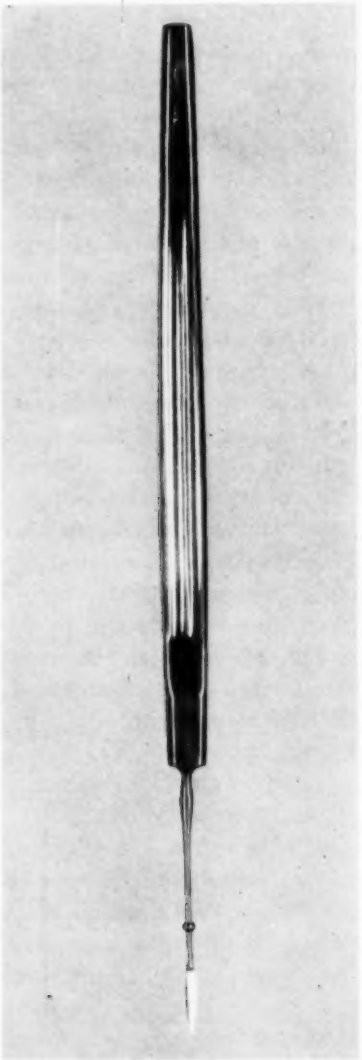

Figure 1. Iris knife used in wheat germ dissections (full size).

one vial, the residues from the same kernels in a second vial. These vials, unstoppered, were placed at once in an air oven maintained at 80° C. for several hours to dry them sufficiently so that spoilage would not occur. They were then corked and placed in a desiccator until a considerable number of dissections had been completed. The vials, with stoppers removed, were then placed in a vacuum oven and dried until they ceased to lose weight. They were then returned to the desiccator, stoppered, allowed to cool, and promptly weighed. The dried germs were weighed to the nearest 0.01 mg. on a micro-balance in the laboratory of the School of Chemistry of the University of Minnesota. This phase of the work was handled by a competent and specially trained micro-analyst. The dried kernel residues (ex-germ) were weighed on a high grade analytical balance to the nearest 0.1 mg.

The sum of the two weights was recorded as the moisture-free weight of the 50 entire wheat kernels, and these weights, divided by 50, were recorded as the average weight per kernel in milligrams. The average weight per germ in milligrams was likewise recorded, and this weight, divided by the weight per kernel and multiplied by 100, represented the percentage of germ. The averages of the two sub-samples taken from each wheat sample are recorded in Table I.

As a crude test of the accuracy of sampling and of germ separation it is interesting to note that the average difference in duplicate determinations was only 0.11% in terms of percentage of germ. This compares favorably with many chemical determinations in which the percentages involved are of the same order of magnitude.

From the data thus computed and recorded it appears that the percentages of germ in the hard red spring and hard red winter wheat samples examined by us are approximately the same. Moreover, they are in the same general range as reported by Fleurent for Canadian wheat (2.05%) and somewhat less than the findings of Percival and Grischenko. It thus appears that the estimate of Ingersoll and Bessey (6%) was substantially in error.

The variability was not large among the four spring wheat samples. We are at a loss to account for the low germ content of one sample of winter wheat, labeled "Turkey, Montana." The kernels in this sample were normal in shape and size, and did not differ widely from the average in kernel weight. In the instance of the other four hard winter wheats the variability was almost identical with that of the spring wheats, and was actually rather small. Moreover the hard winter wheats were very similar to the hard spring wheats in average germ content.

As a matter of added interest, two lots of kernels were separated by hand picking from the "Turkey, Montana" sample on the basis of

relative kernel size, and designated as "large" and "small" kernels. The germs were dissected from these two lots, and it developed that the percentage of germ was not greatly different in the instance of large and small kernels. Scanning the data resulting from the determinations on the hard wheats (spring and winter combined) indicates

TABLE I

GERM CONTENT OF HARD, SOFT AND DURUM WHEATS

Description of wheat and source	Average dry weight per kernel	Average dry weight per germ	Average germ
	Mg.	*Mg.*	%
Hard Red Spring			
Canadian No. 1 Hard, Ft. William, 1935 crop	25.81	0.582	2.26
Thatcher, University Farm, St. Paul, 1935 crop	20.88	0.509	2.44
Ceres, North Dakota, 1934 crop	27.37	0.603	2.21
Minnesota and North Dakota, 1935 crop	15.79	0.362	2.30
Average for Hard Red Spring	22.46	0.514	2.29
Hard Red Winter			
Oklahoma, 1936 crop	23.89	0.586	2.45
East North Central Texas, 1936 crop	23.58	0.522	2.21
No. 2 Hard, Nebraska, 1935 crop	18.74	0.412	2.20
Turkey, Montana	23.78	0.413	1.74
Large kernels } Separated from above	33.25*	0.578*	1.74*
Small kernels } Separated from above	21.56*	0.400*	1.85*
Minturki, 1935 crop	21.73	0.500	2.30
Average for Hard Red Winter	22.34	0.487	2.18
Average for all hard wheats	22.39	0.499	2.23
Durum			
Mindum, St. Paul, Minnesota	32.12	0.895	2.79
Canadian, 1934 crop	32.25	1.033	3.20
Canadian, elevator mixture	29.40	0.915	3.11
Canadian	20.18	0.543	2.69
Canadian, 1935 crop	19.02	0.516	2.71
North Dakota	26.48	0.714	2.70
Elevator lot	27.09	0.742	2.74
Kubanka, Crookston, Minnesota, 1934 or 1935 crop	32.70	1.088	3.33
Average for Durum wheats	27.40	0.806	2.94
Soft Wheats			
No. 2 Soft White, 59.7 lbs., Spokane, Washington, 1934 crop	35.14	1.002	2.85
Soft Red Winter, Illinois, Indiana, Ohio, 1935 crop	24.97	0.593	2.37
Hybrid No. 128, Pullman, Washington, 59.5 lbs.	23.67	0.632	2.67
Average for soft wheats	27.93	0.742	2.66
Average for all wheats	25.30	0.662	2.61

* Omitted from average.

that there is no direct relationship between kernel weight and percentage of germ. This was further supported by computing the coefficient of correlation of these two variables which was $r = -0.184 \pm 0.149$. The correlation is small, and moreover is only slightly more than the probable error which renders it of doubtful significance. It is recog-

nized, to be sure, that the number of entries (nine) is too small for reliable computation in such instances.

In the case of the eight durum samples there is an indication of a positive relationship between kernel weight and percentage of germ, however, and the value of $r = +0.709 \pm 0.185$ gives evidence of a trend toward a larger germ in the heavier grains. The number of comparisons is too small, however, to establish this as a general principle.

It does appear that the durum germs are substantially larger than those of the *vulgare* (spring and winter wheats), and also constitute a larger percentage of the total kernel. The average of the three soft wheats was intermediate between the *vulgare* and durum, but they were so variable, ranging from 2.37% to 2.85%, and the numbers are so few as to make detailed comparisons impracticable.

Summary

Vulgare wheat samples of the hard spring and hard winter classes were rather uniform in percentage of germ in their tissues, and averaged 2.23%.

Durum wheat kernels had substantially heavier germs and thus had a higher average germ content, namely 2.94%.

Soft wheats examined were approximately intermediate between the *vulgare* and winter wheats.

The average germ content of 20 wheat samples was 2.67%.

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AN IMPROVED METHOD OF SUGAR DETERMINATION IN DIASTATIC ACTIVITY MEASUREMENTS[1]

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(Read at the Annual Meeting, May 1937)

The study of factors affecting diastatic activity and the control of such activity have in recent years become of such interest to cereal chemists that considerable effort has been expended in the development of suitable methods of determination. It is not our purpose to present here an extended review of the various methods which have been and are now in common use. It is significant to note, however, the large number of methods available, indicating not only the importance of such procedures, but also that no one method is sufficiently free from objections to have become universally adopted.

Recently Blish and Sandstedt (1933) and Putnam, Blish, and Sandstedt (1935) presented a procedure for the determination of diastatic activity in which the measurement of sugar concentration is based on the micro blood sugar technique developed by Hagedorn and Jensen (1923). This procedure, dependent on measurement of the unreduced ferricyanide from a known excess of that reagent, has been widely adopted by cereal workers because it offers several advantages over the older copper reduction methods. Since that time, a number of studies, of which those of Miller and Van Slyke (1936) and Whitmoyer (1934) should be mentioned in particular, have appeared in which the authors have reported improvements on the original Hagedorn-Jensen ferricyanide method.

Of the various methods advocated, that proposed by Miller and Van Slyke (1936) appeared to be best suited to modification to meet the requirements of a diastatic activity determination. Their procedure may be briefly summarized as follows: To the solution of reducing sugar is added an alkaline ferricyanide reagent containing a comparatively large and not necessarily accurately measured excess of ferricyanide. This mixture is placed in a boiling water bath for 20 minutes, cooled, and washed into the titration vessel with dilute sulfuric acid. The ferrocyanide formed by the reducing action of the sugar is titrated directly with a standard ceric sulfate solution, using an inside oxidation-reduction indicator.

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We have revised this method to make it applicable to diastatic activity determinations, and in so doing have included a few minor changes which offer several improvements. The procedure, described in complete detail below, involves no new theoretical principles, but rather represents a combination of the best features of several methods in one determination which is rapid, convenient and accurate.

Procedure

A. Reagents

1. Alkaline ferricyanide.

   Forty grams $K_3Fe(CN)_6$, 100 g. $Na_2HPO_4 \cdot 12H_2O$, and 12 g. NaOH per liter of solution. These materials need not be weighed more accurately than ± 0.02 g. This reagent deteriorates slowly in the presence of light and preferably should be stored in a dark bottle.

2. Ceric sulfate.

   Approximately 100 g. of $Ce(SO_4)_2$[2] placed in a 1-liter beaker with 35 c.c. of concentrated H_2SO_4 and 35 c.c. of H_2O. The mixture is heated and stirred with the addition of more water in small increments until nearly all of the $Ce(SO_4)_2$ is dissolved. It is then filtered, cooled, and made up to 1 liter. The solution is standardized against Mohr's salt as described below, and diluted to give a final concentration of exactly 0.176 N.

   From this stock solution, 100 c.c. are diluted to 1 liter, with the inclusion of 10 c.c. of concentrated H_2SO_4, to give the solution for titration. This reagent thus is 0.0176 N with respect to $Ce(SO_4)_2$ and approximately 0.50 N with respect to H_2SO_4.

3. Dilute sulfuric acid.

   One hundred cubic centimeters of concentrated H_2SO_4 diluted to 1 liter, giving a final concentration of 3.58 $\pm$ 0.05 N.

4. Indicator.

   Approximately 100 mg. of Setopaline C[3] dissolved in 100 c.c. of water.

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[2] Commercial product purchased from G. Frederick Smith Chemical Co., Columbus, Ohio.

[3] This dye, the chemical constitution of which has not been disclosed, may be obtained from Eimer and Amend, New York. The indicator has an oxidation-reduction potential of + 1.1 volts at pH 1.0.

B. Standardization of ceric sulfate

It is convenient in the standardization of the ceric sulfate stock solution to use a Mohr's salt of high purity as a primary standard.[4] A 0.10 N solution is prepared containing 39.21 g. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 25 c.c. concentrated H_2SO_4 per liter. Fifty cubic centimeters are pipetted into a titration vessel, 25 c.c. of the dilute sulfuric acid solution and 10 drops of indicator are added, and the Fe^{++} titrated with the stock ceric sulfate solution. The color changes sharply from a bright yellow-green to a golden brown, easily recognized against a white background.

The normality of the ceric sulfate solution is calculated from the expression

$$N = \frac{\text{c.c. Mohr's salt}}{\text{c.c. } Ce(SO_4)_2} \times 0.1000.$$

With the volumes given above, this becomes

$$N = \frac{5.000}{\text{c.c. } Ce(SO_4)_2}.$$

From this preliminary standardization, the permanent stock solution is prepared by accurate dilution to a concentration of 0.176 N.

C. Determination of reducing sugars

A 5 c.c. aliquot of the flour-buffer digestion mixture, inactivated and clarified in the usual manner (Blish and Sandstedt, 1933), is accurately pipetted into a large test tube, 10 c.c. of the alkaline ferricyanide reagent added, and the tube placed in a boiling water bath for exactly 20 minutes. At the end of this time, the tube is rapidly cooled, either under running water or by placing it in a large bath of cold water for 2 or 3 minutes. The contents of the tube are then poured into a 125 c.c. Erlenmeyer flask or other suitable titration vessel, using 25 c.c. of the dilute sulfuric acid reagent in two portions to rinse the tube. Ten drops of Setopaline C indicator are added and titration with ceric sulfate carried out as described under the standardization procedure. To obtain the diastatic activity of the sample, expressed as milligrams maltose per 10 g., subtract the volume of ceric sulfate in cubic centimeters used in the blank from that of the sugar determination and multiply the difference by 10.

[4] A grade of Mohr's salt designated as "Analytical Reagent, Low Manganese" obtained from Mallinckrodt Chemical Co., St. Louis, has been found to be entirely satisfactory.

D. Blank determination

Since there may be small amounts of ferrocyanide present in the ferricyanide used in reagent 10 above, and since the reagent itself is somewhat subject to deterioration, it is well in precise work to correct for these sources of error. A blank determination should be carried out, using 5 c.c. of a mixture of 46 c.c. acetate buffer, 2 c.c. of sodium tungstate solution and 2 c.c. of 10% sulfuric acid to replace the 5 c.c. of filtrate from the digestion mixture. The titration value thus obtained, which should not exceed 0.5 to 0.7 c.c. of ceric sulfate, should be deducted from the volume of ceric sulfate used in the actual determination.

Discussion

Since the procedure described is entirely empirical, it is vital to follow exactly the conditions prescribed in order to obtain accurate results.

Reagents: Experiments have shown that the oxidation of sugar solutions by an alkaline ferricyanide reagent is, like other sugar oxidations (see Caldwell, Doebbeling, and Manian, 1936), appreciably affected by the pH of the reaction system. To minimize the effect of small variations in alkalinity, we have used NaOH and Na_2HPO_4 as the alkalizing agent rather than Na_2CO_3, utilizing the buffering effect of the former system.

Addition of sulfuric acid in the preparation of the dilute ceric sulfate solution is necessary to prevent hydrolysis, with resultant formation of insoluble ceric hydroxide or hydrous ceric oxides. Under the conditions prescribed we have found that the titration solution is entirely stable over periods up to four months' time. We have been unable to detect any change in the concentration of the stock ceric sulfate solution over the same length of time.

The dilute sulfuric acid solution, if it is only to be used to acidify the reaction mixture before titration, need not be carefully made up. We have found it convenient, however, to use the same solution in the inactivation of the flour-buffer digestion mixtures, and accordingly are quoting the specifications for this reagent given by Putnam, Blish, and Sandstedt (1935).

The indicator deteriorates somewhat with age, and the end-point becomes less distinct. We have experienced no difficulty in recognizing the end-point when using indicator solutions three or four weeks old, but operators unfamiliar with the titration may at first find it advisable to make up a fresh indicator solution each week.

Standardization: Other methods may be employed for the standardization of the ceric sulfate solution (see Miller and Van Slyke, 1936).

We have, however, found the procedure described above to be simple and reliable.

Determination of reducing sugars: The volume of sugar solution taken for analysis may be reduced if the maltose content of the sample is above 700 mg. per 10 g. In such cases, the aliquot taken should always be diluted to 5 c.c. In the calculation of maltose from the volume of ceric sulfate used, the factor 10 should be replaced by the factor appropriate to the particular aliquot of sugar solution taken.

Accuracy and range of the method: The procedure described has been carefully checked against solutions containing known amounts of maltose. A sample of commercial maltose was found to have a reducing power equivalent to 92.1% anhydrous maltose when determined by

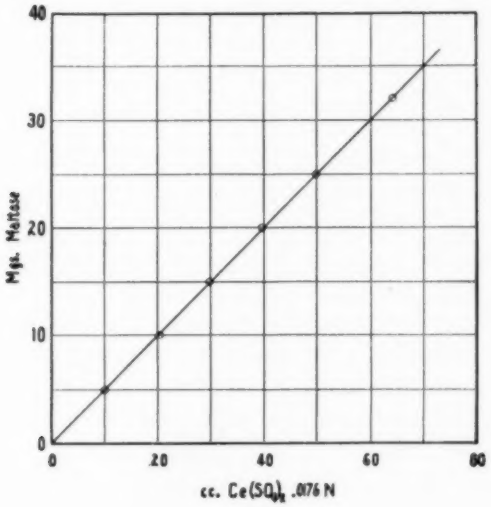

Fig. 1

the Quisumbing-Thomas copper reduction method. A known weight of this material was dissolved in a mixture of acetate buffer, sodium tungstate solution, and dilute sulfuric acid in the proportions present in an inactivated digestion mixture. Analyses of several such known maltose solutions, which thus reproduce the conditions of an actual diastatic activity determination, were made by the ceric sulfate method. The results, which are shown graphically in Figure 1, indicate that the relation between maltose concentration and ceric sulfate is a linear function up to a maltose concentration of 35 mg. per 5 c.c., which corresponds to a diastatic activity value of 700.

Comparative determinations by the Blish-Sandstedt and ceric sulfate methods were made on samples of six different flours. Aliquots

of the same digestion mixture were taken in the case of each flour, and determinations by each method were made in duplicate. The results, which are shown in Table I, indicate that the agreement between the two methods is excellent. Duplicate determinations by each method agreed to within approximately 0.5%.

TABLE I

COMPARATIVE DETERMINATION OF DIASTATIC ACTIVITY BY BLISH-SANDSTEDT AND $Ce(SO_4)_2$ METHODS

Flour number	Diastatic activity	
	Blish-Sandstedt	$Ce(SO_4)_2$
6400	182	182
6987	141	139
6990	213	211
6993	215	212
6997	290	293
6999	222	227

Advantages of the method: We believe the proposed ceric sulfate method offers substantial advantages in point of speed and convenience over the Blish-Sandstedt procedure. Since the range of maltose concentration which can be measured is nearly twice that of the Blish-Sandstedt method, the necessity for dilution of digestion mixtures is correspondingly less frequent. There is one less reagent to prepare and handle, and the preparation of the alkaline ferricyanide reagent has been much simplified, since there is no need for standardization. Because reduced ferricyanide is determined by direct titration rather than by difference, there is no necessity for extreme care in the amount of ferricyanide reagent added. The relation between maltose concentration and ceric sulfate used is a strictly linear function over the range 0 to 35 mg. of maltose per 5 c.c.; therefore a conversion table need not be used.

In the lower ranges of maltose concentration there seems to be little or no difference between the two methods as to accuracy and precision. Above a maltose concentration of 17 mg. per 5 c.c., the ceric sulfate method should be the more accurate, since the total maltose is determined directly rather than by dilution and the use of a multiplication factor.

Other applications: We have found the method entirely satisfactory in a number of determinations other than that of the amylase activity of flour. The activity of other amylase materials may, of course, be determined, and with suitable standardization other sugars may be determined as well. We have applied the method to the determination

of glucose with no difficulty. Despite the fact that Miller and Van Slyke report unsatisfactory results with fructose, we have been reasonably successful in the determination of invert sugar and of sucrose by inversion with this procedure. Preliminary results indicate that under the conditions specified, 1 c.c. of 0.0176 N ceric sulfate is equivalent to 0.466 mg. glucose and 0.462 invert sugar.

Summary

A modification, based on the micro sugar method of Miller and Van Slyke, of the Blish-Sandstedt diastatic activity procedure has been described. The method depends on the direct titration of ferrocyanide, formed by sugar reduction, with a standard ceric sulfate solution, using Setopaline C as an inside oxidation-reduction indicator.

The method proposed has a marked advantage over the Blish-Sandstedt procedure in point of time and convenience and also offers a substantial increase in accuracy with higher concentrations of maltose.

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A SHORT GASSING POWER METHOD

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Sandstedt and Blish (1934) have shown that in order to reduce yeast variability to a minimum in gassing power tests, it is necessary to use small quantities of yeast. This necessitates 4 hours to 6 hours for a gassing power determination. A reduction in the time required for the gassing power determination would be of great advantage in the mill control laboratory.

Blish and Sandstedt (1937) report that the rate of fermentation of maltose by fresh baker's compressed yeast can be increased enormously by the use of maltose fermentation activators. The most potent of these activators was prepared by drying "filler-free" (starch-free) baker's compressed yeast at low temperatures. A convenient method is to crumble the "filler-free" yeast, spread it over a flat surface, and dry it by use of an overhead heater and a current of air from a fan.

Preliminary experiments using this activator with fresh yeast showed that the rate of fermentation could be increased enough to shorten materially the time necessary for a gassing power determination, and that 0.5 g. of fresh yeast and 0.5 g. of yeast activator acting on a paste made with 10 g. of flour gave the most rapid fermentation with the minimum of error due to yeast variability. These quantities of ingredients gave results that could be checked from day to day in the same laboratory and gave concordant results between this laboratory and the Western Star Mill laboratory (Claude F. Davis).

Figure 1 shows typical gassing power curves of a series of flours using 0.3 g. of fresh yeast on a dough made of 10 g. of flour in the Sandstedt-Blish pressuremeter. Figure 2 shows the gassing power curves of this same series of flours using 0.5 g. of yeast and 0.5 g. of activator. It is seen that both methods give the same differentiation between flours and the same type of curve for each flour.

If the pressure readings are taken at the end of 2 hours and 3 hours, figures are obtained which indicate for most practical purposes the gassing-power potentialities of each flour. The 2-hour reading gives much the same information that is obtained from the combined maltose and sucrose values; *i.e.*, the quantity of readily available starch and sucrose.

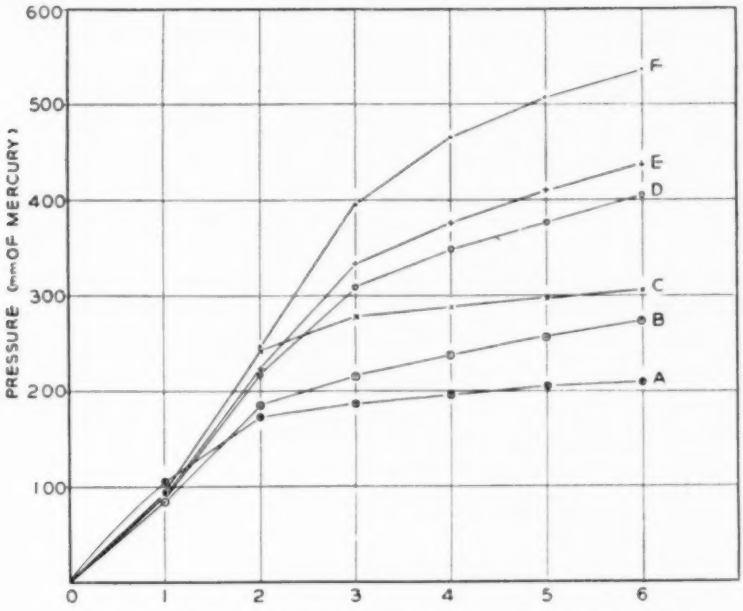

Figure 1. Gassing power curves of flour using 0.3 g. of yeast time (hours).

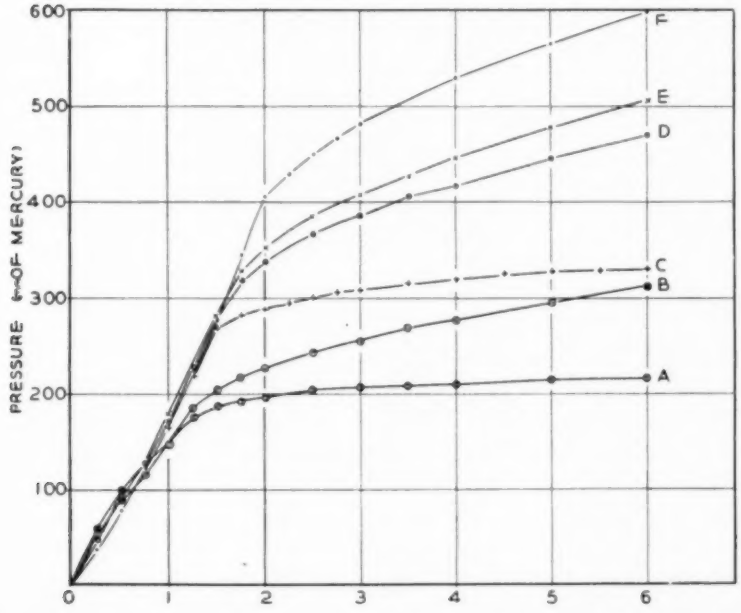

Figure 2. Gassing power curves of flours using 0.5 g. of yeast and 0.5 g. of activator time (hours).

The gain during the third hour gives a value indicative of the ability of the flour to sustain gas production over a long period. As may be seen from an examination of Figure 2, the reading at two hours is not always indicative of the ability of the flour to maintain gas production over a long period of time, hence the necessity for the 3-hour reading.

Adopted Procedure

The procedure as adopted is as follows: A suspension is made containing 0.5 g. of fresh baker's compressed yeast and 0.5 g. of dried "filler-free" yeast in 10 c.c. volume. This suspension is placed in the 30° C. water bath for one hour to ferment out the blank in the activator. A paste is made, in the pressuremeter, using 10 c.c. of the yeast activator suspension on 10 g. of flour. The procedure from the time of mixing the paste is the same as in the 4-hour to 6-hour method for determining gassing power (Sandstedt and Blish, 1934) except that the pressure readings are taken at the end of 2 hours and 3 hours.

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MECHANICAL SHAKING DEVICE[1]

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In the course of various investigations in this laboratory, we have required a mechanical shaker (see Figure 1) which could be operated either continuously or intermittently in a constant temperature water bath. It was also desirable to have an apparatus sufficiently flexible in design to permit the use of flasks of several different sizes. We are here describing a shaker constructed to meet these requirements, hoping that others may find the information useful.

The main portion of the shaker, as will be seen from the accompanying illustration, is composed of a brass angle frame reinforced at the corners with brass blocks. Horizontally through these blocks are

[1] Paper No. 10, Journal Series, General Mills Research Laboratories.

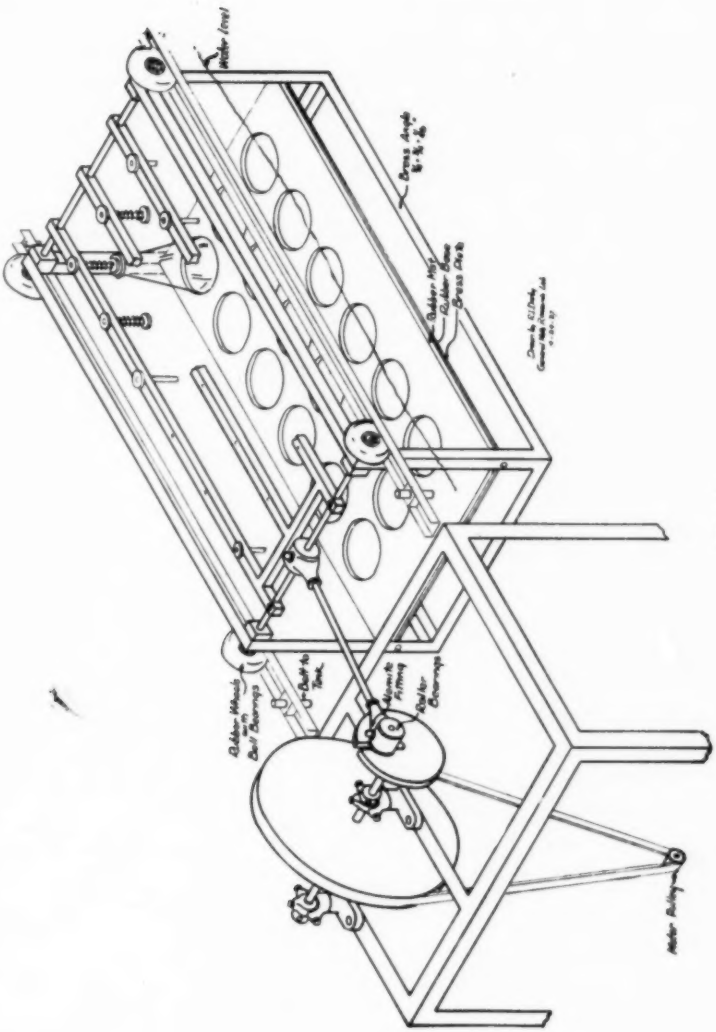
Water Level
Brass Angle
Rubber Strip
Rubber Band
Brass Plate
Rubber Wheels
Ball Bearings
Rubber Bearings
Motor Pulley

Fig. 1.

two steel axles on which are mounted ball bearing rubber wheels operating in channel iron tracks suspended over the constant temperature bath. Approximately three-quarters of the frame is below the surface of the water. The bottom, constructed of sheet brass covered with two layers of corrugated rubber matting, is bolted to the frame. It may be raised or lowered to accommodate varying sizes of flasks. Spring plungers passing through brass bars which parallel the long dimension of the frame hold the flasks in place by pressure on the stoppers. Additional security is given by cutting holes in the upper layer of rubber matting to fit the bases of the flasks.

The frame is driven by a connecting rod from the center of one axle to an eccentric, which in turn is mounted on a jack-shaft with a pulley of size sufficient to give approximately 100 r.p.m. The eccentric is slotted so that the distance from the connecting rod head to the shaft may be changed at will, thus varying the stroke of the frame. It has been found that with only a few flasks in the shaker there is a tendency to splashing, due to the formation of a standing wave in the water. This situation has been handled satisfactorily by placing an inverted brass trough around the inside of the bath to serve as a splash rail.

The shaker may be operated continuously as described, or intermittent agitation may be obtained by adding a regulating system constructed from a clock, a relay and a source of current (dry cells or small transformer). The clock is easily converted to a time switch. The moving contact is made by soldering a thin strip of spring brass to the minute hand, and the stationary points are of brass strips mounted on an insulated wire fastened to the clock face. The length of the stationary contacts will, of course, determine the time interval during which the shaker will operate. This time switch actuates the relay, which in turn opens and closes the circuit through the shaker motor.

This shaker has been in operation in our laboratory for nearly a year, and has given entirely satisfactory service.

We wish to thank R. I. Derby for preparing the drawing shown above.

DR. D. A. COLEMAN

1892 ~ 1938

Editor-in-chief of Cereal Chemistry

1931 ~ 1938